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Doctoral Programme in Oral Science (FINDOS-Helsinki).

The Oral Epithelial Cell: An Active Player in Recurrent Aphthous Ulcers

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Academic Dissertation

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in Lecture Hall 3 at Biomedicum Helsinki 1, Haartmaninkatu 8, Helsinki, on June 12th 2015, at 12 o'clock noon.

Helsinki 2015

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ISBN 978-951-51-1255-2 (paperback)
ISBN 978-951-51-1256-9 (PDF)
ISSN 2342-3161
http://ethesis.helsinki.fi

Hansaprint Oy
Helsinki 2015

“Success is Getting What You Want;
Happiness is Wanting What You Get”
- Dale Carnegie

To the late Professor Yrjö T. Konttinen

Contents

List of Original Publications

This thesis is based on the following original publications, which are reffered to in the text by their Roman numerals.

I. Al-Samadi A, Drozd A, Salem A, Hietanen J, Häyrinen-Immonen R, Konttinen Y. Epithelial cell apoptosis in recurrent aphthous ulcers, J Dent Res 2015 Apr 10. pii: 0022034515581012. [Epub ahead of print]

II. Hietanen J, Häyrinen-Immonen R, Al-Samadi A, Trokovic N, Koskenpato K, Konttinen YT. Recurrent aphthous ulcers – a Toll-like receptor mediated disease? J Oral Pathol Med 41: 158–164, 2012.

III. Al-Samadi A, Kouri V-P, Salem A, Ainola A, Kaivosoja M, Barreto G, Konttinen YT, Hietanen J, Häyrinen-Immonen R. IL-17C and its receptor IL-17RA/IL-17RE identify human oral epithelial cell as an inflammatory cell in recurrent aphthous ulcer, Oral Pathol Med 43:117–124, 2014.

IV. Al-Samadi A, Salem A, Ainola M, Hietanen J, Häyrinen-Immonen R, Konttinen Y. Increased beta 2 defensin in recurrent aphthous ulcer, Oral Dis 21:292–8, 2015.

List of Abbreviations

4HNE	4-hydroxynonenal
AMP	Antimicrobial peptide
AP-1	Activator protein 1
BD	Beta defensin
CD8	Cytotoxic T cell
CXCL12	C-X-C motif chemokine 12
CXCR4	C-X-C chemokine receptor type 4
DAB	Diaminobenzidine tetrahydrochloride
DAMP	Damage-associated molecular patterns
DAPI	4'6-Diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DS-HMGB1	Disulphide-HMGB1
EBV	Epstein–Barr virus
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
HCMV	Human cytomegalovirus
HHV	Human herpes virus
HIV	Human immunodeficiency virus
HKLM	Heat-killed Listeria monocytogenes
HLA	Human leukocytes antigen
HMGB1	High-mobility group box 1
HOK	Human oral keratinocytes
HPV	Human papilloma virus
HSP	Heat shock protein
HSV	Herpes simplex virus

IFN-γ	Interferon gamma
IL	Interleukin
LPS	Lipopolysaccharide
LSD	Least significant difference
LTA	Lipoteichoic acid
MAMP	Microbe-associated molecular patterns
MCT	Mast cell tryptase
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa-light chain enhancer of activated B cells
NK	Natural killer
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PRR	Pathogen recognition receptor
qPCR	Quantitative real-time PCR
RAU	Recurrent aphthous ulcer
RBC	Red blood cell
ROS	Reactive oxidative species
RPLP0	Ribosomal protein large P0
RT	Room temperature
SLO	Soybean lipoxygenase
SLS	Sodium lauryl sulphate
TGF-α	Transforming growth factor alpha
Th	T-helper
TIR	Toll-IL-1 receptor
TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein

TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adapter-inducing interferon β
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VZV	Varicella zoster virus

Abstract

Recurrent aphthous ulcer (RAU) is an ulcerative disease of the oral mucosa characterised by the appearance of ulcerations in the oral mucosa accompanied by an erythematous halo area surrounding the ulcer and showing signs of acute inflammation.

While RAU affects approximately 20% of the population globally, its pathogenesis remains poorly understood. Furthermore, most studies concentrate on treatment while few address the pathogenesis of the disease.

This project aimed to determine the mechanisms of oral epithelial cell death in RAU, the role of these cells in disease pathogenesis in terms of toll-like receptor (TLR) expression, and the ability of the these cells to produce chemokines, pro-inflammatory cytokines, and antimicrobial peptides. Together these may first aggravate and, then, down-regulate the inflammation and initiate the healing process.

For this purpose, we collected 13 aphthae and 11 healthy control biopsies for immunohistochemical staining, immunofluorescence staining, and quantitative PCR. For functional studies, we cultured primary oral keratinocytes and oral squamous cell carcinoma cell-line SCC-25 and tested their responses to different stimuli.

Our results highlight the importance of oral epithelial cells in RAU; interestingly, oral epithelial cells in RAU tested positive for apoptosis markers caspase-3, especially at the superficial and spinous layer, and TUNEL, but negative in controls. We also found that TLRs are primarily present in the basal and suprabasal layers of control epithelium, but their expression extends to the superficial layer in RAU epithelium. Additionally, we found significally higher expressions of tumour necrosis factor-α (TNF-α), interleukin-8 (IL-8), IL-17C, and beta defensin 2 (BD-2) in RAU oral epithelium compared with control epithelium.

Functional studies on cultured primary oral keratinocytes and SCC-25 supported our results from RAU biopsies since these cells responded to damage-associated molecule patterns (DAMPs), such as self-DNA and pro-inflammatory cytokines including IL-17C, TNF-α, and interferon gamma (IFN-γ), through a significant increase in the expression of selected molecules including TLR2, TNF-α, and BD-2.

Based on our findings, RAU may begin with a strong initiating factor activating a self-amplificatory cycle. This cycle is characterised by the induction of epithelial cells apoptosis at the superficial layer down to the basal layer, a change in the pattern of TLR distribution, the up-regulation of several chemokines and pro-inflammatory cytokines, and, finally, the secretion of antimicrobial peptides initating the healing process. As a result of the lack of adaptive immunity in RAU, the cycle recurs when the mucosa is subjected to an initiating factor of the same sequence.

1. Introduction

Oral ulcerative diseases represent a group of mucosal disorders which affect oral mucosa; the most common type of oral ulcerations are recurrent aphthous ulcers (RAUs) or (canker sores). The word aphthous (meaning 'ulcer' in Greek) was first used by Hippocrates (460–370 BC) to describe ulcerations in the oral mucosa. Canker sores, which is Latin in origin, was also used at that time.

Despite centuries of knowledge about RAUs, which currently affect approximately 20% of the population, their origin remains unknown. Even after thousands of studies beginning with an article published by Mikulicz and Kümmel in 1898, the exact reason for the appearance of RAU remains elusive. Based on these studies, however, scholars suggested several aetiological factors that play a role in RAU onset. Some scholars argued that RAUs serve as a sign of other systemic diseases such as Behçet's disease.

RAU represents an acute inflammation usually affecting non-keratinised oral mucosa, such as the tongue, labial, and buccal mucosa, RAU can also affect keratinized oral mucosa such as palate and attached gingiva although such instances are uncommon.

A RAU lesion is a self-healing ulceration; the ulcer appears within a few hours and persists for 5 to 10 days in a minor form, during which time the inflammation subsides and the ulcer heals. During the ulcer episode, the patient suffers from severe pain and difficulties eating, speaking, and swallowing especially if the ulcer is located on the tongue.

A RAU lesion is characterised by the complete loss of the epithelium, which appears clinically as white or gray in colour and is surrounded by a red erythematous halo area, possibly indicating the severity of the inflammation in the area. As a result of the acute inflammation, a RAU lesion is highly infiltrated by several types of inflammatory cells, such as neutrophils, lymphocytes, macrophages, and others, which may at first fight microbes that have easy access to

the oral mucosa through the unprotected ulcer area. Second, the inflammation may increase, indicating the first step in healing.

Since the initial cause of disease remains unknown, the reasons behind the quite rapid ability of the oral mucosa to repair after an acute inflammation remain unclear.

Because immune cells are the primary producers of cytokines and chemokines, most studies on RAU addressed the role of immune cells in pathogenesis. Few studies addressed the role of oral epithelial cells in RAUs and the possible causes for epithelium loss.

Here, we suggest that oral epithelial cells play an active role in RAUs through the release of a cocktail of alarmins, chemokines, cytokines, and antimicrobial peptides which work together to up-regulate the inflammation and initiate the healing process.

2. Review of the literature

2.1. Definition of RAU

RAU is an acute inflammatory ulcerative disease that affects the oral mucosa characterised by recurrent episodes of oral ulcerations accompanied by severe pain affecting the everyday lives of patients (Scully and Porter, 2008).

2.2. Classification of RAU

Three forms of RAUs exist, depending on the size of the ulcer, the number of ulcers per episode, the duration of healing, and the ability to heal without scar formation. The three forms are as follows.

Minor RAU represents the most common form , comprising 80% of all RAU cases. Characteristics of minor RAU include their small size (less than 10 mm) and one or multiple (up to five) ulcers primarily affecting non-keratinised oral mucosa or the palate and attached gingiva in rare cases. The ulcer is painful and takes seven to ten days to heal spontaneously without scars forming. This type of ulcer normally affects children and young people (Figure 1).

Major RAU represents the second type, regarded as the severest type of RAU. These types of ulcers normally appear larger than 10 mm and penetrate deeper than minor RAU, leading to severe pain and a longer healing period of six weeks or more. Scar formation normally accompanies healing. Major RAUs usually occur after puberty.

Herpetiform RAUs are characterised by multiple (up to 100 or more), small (2 to 3 mm) ulcers sometimes fused to form a large ulcer. The herpetiform RAU took its name from the herpes simplex virus (HSV) because of its resemblance to oral symptoms. Herpetiform RAUs take seven to ten days to heal without scar formation.

Figure 1. Minor RAU of the labial mucosa (Scully and Felix, 2005).

2.3. Epidemiology of RAU

While no population-based studies on the prevalence and incidence of RAU exist, reports from various countries showed that their prevalence varies geographically. RAU seems uncommon among Bedouin Arabs (Fahmy, 1976) and more frequent in Western European countries and in North America (Embil et al., 1975). Others found that RAU affects 5% to 66% of the the population (Fahmy, 1976; Miller et al., 1980), with a global estimated prevalence of 20% (Axell and Henricsson, 1985; SIRCUS et al., 1957). The prevalence of RAU in selected countries is presented in table 1. With regards to gender, some studies found that RAU affects more females than males (Pongissawaranun and Laohapand, 1991; SHIP et al., 1961), particularly among children (Field et al., 1992), while other studies found no gender difference (Miller et al., 1980). Saraceno et al reported recently that RAU affects 43% of Italians ageing between 2 and 17 years (Saraceno et al., 2015).

RAU affects students and individuals from a higher socioeconomic class (Crivelli et al., 1988; SHIP et al., 1961; Ship, 1966). The chance of one's offspring acquiring RAU increases by 63.3% to 90% if both parents experience RAU. Additionally, children with a family history suffer from RAU at an earlier age and

experience stronger RAU episodes (Miller and Ship, 1977; Miller et al., 1980; Ship, 1965).

Table 1. Prevalence of recurrent aphthous ulcers in selected countries.

RAU prevalence (%)		Population studies			References
Clinical examination	Patient's history	Country	Number of participants	Age of participants	
1.2		USA	7,785	17-39	(Rivera-Hidalgo et al., 2004)
1.03		USA	33,994	<1-≥60	(Chattopadhyay and Chatterjee, 2007)
1.2		Turkey	765	5-95	(Mumcu et al., 2005)
1.4	18.3	Germany	655	35-44	(Reichart, 2000)
	9.7	Slovenia	555	15-65	(Kovac-Kovacic and Skaleric, 2000)
	78.1	Jordan	684	13-68	(Safadi, 2009)
2.0	27.3	Poland	814	14-18	(Gorska, 1997)

Modified from Slebioda et al. (Slebioda et al., 2014).

2.4. Predisposing factors for RAU

Table 2 summarises the factors predisposing individuals to RAU (page 14).

Age and sex

RAU episodes begin at a young age, whereby 40% of children 15 years old or younger suffer from RAU, with the first ulcer appearing before the age of 5 years (Miller et al., 1980). A minor RAU normally appears in the second decade of life, while a hepitiform RAU appears in the third decade of life (Scully and Porter, 1989). Some studies found that females (both adults and children) are more predisposed to RAU than males, while others found no gender difference (Miller et al., 1980; Pongissawaranun and Laohapand, 1991; SHIP et al., 1961). Axéll and Henricsson (1985) found that RAU episodes decrease in both males and females with an increasing age among a Swedish population (Axell and Henricsson, 1985), while Sircus et al. found that such a decrease occured only in males in a Scottish population (SIRCUS et al., 1957).

Heredity

When both parents experience RAU, children have a 63.% to 90% chance of experiening RAU compared to 20% of children with no family history of RAU (Ship, 1972). Children with a family history of RAU suffer from RAU episodes earlier than others, and the episodes are normally more severe in these children (Miller and Ship, 1977; Miller et al., 1980; Ship, 1965). Several studies found an association between RAU and genetically specific human leukocytes antigen (HLA) subtypes among different ethnic groups.

RAU incidence was associated with an increase in HLA2 (Challacombe et al., 1977), B12 (Lehner et al., 1982; Malmstrom et al., 1983), HLA-B51 in Korean and Jewish populations, Cw7 in Jewish populations (Chang et al., 2001; Shohat-Zabarski et al., 1992), DR2 (Lehner et al., 1982), DR4 in Turkish populations (Ozbakir et al., 1987), DR5, A28 in Greek populations (Albanidou-Farmaki et al., 1988), DRw9 in Chinese populations (Sun et al., 1991), DR7, and MT3 in Sicilians (Gallina et al., 1985; Gallina et al., 1985).

Other studies found an inverse relationship between some genes and RAU, such as HLA-B5 in Sicilians (Gallina et al., 1985) and DR4 in Greeks (Albanidou-Farmaki et al., 1988).

Furthermore, a single nucleotide polymorphism for a high tumor necrosis factor (TNF)-α and interleukin (IL)-β production is associated with a high prevalence of RAU in a Brazilian population (Guimaraes et al., 2006; Guimaraes et al., 2007).

Genetic studies found some inconsistencies which may result from differences in the ethnic groups studied.

Hormonal disturbance

While researchers have studied the relationship between a hormonal disturbance and RAU intensively, no clear results were found. McCartan and Sullivan completed one of the most important reviews in this field (McCartan and Sullivan, 1992) covering all articles published from 1942 to 1991. In this review, the authors concluded that no clear relationship between RAU and the premenstrual period, menopause, or pregnancy exists. They suggested that this may result from an improper reseach design or too few study participants. Yet, some conflicting results from the review exist, such as the remission of RAU during pregnancy (Bishop et al., 1967; SIRCUS et al., 1957), no men over 50 years old develop RAU, and only 10% of women experienced their first episode of RAU between the ages of 50 and 59, which may represent a symptom for another disease entirely (SIRCUS et al., 1957). In addition, 30% of 40 young women selected for one study experienced RAU a few days prior to menstruation (Balan et al., 2012).

Haematological disturbance

Haematological disturbances may represent an important predisposing factor for RAU. The overall frequency of hematinic deficiency was significantly higher in RAU inidividuals than in healthy controls (Compilato et al., 2010; Lopez-Jornet et al., 2014). While some studies found that serum vitamin B12, serum ferritin, haematocrit, and red blood cell (RBC) folate were significantly lower in RAU individuals compared with healthy controls (Khan et al., 2013; Piskin et al., 2002). Contradictory to that, Thongprasom and coworkers showed that only RBC folate was significantly lower in RAU individuals with no significant difference found in serum vitamin B12, hemoglobin, haematocrit, and mean corpuscular hemoglobin concentrations (Thongprasom et al., 2002). Additionally, the daily intake of vitamin B12 and folate in RAU patients was significantly lower than that in healthy individuals (Kozlak et al., 2010). Replacement therapy using vitamins served as an

effective method to treat RAU, whereby vitamins B1, B2, and B6 and folic acid significantly decreased RAU episodes and led to complete remission in some cases (Nolan et al., 1991; Volkov et al., 2009). RAU patients suffering from vitamin B12 and folic acid deficiencies responded to replacement therapy better than those with an iron deficiency (Wray et al., 1975).

Even with such promising results, vitamin deficiencies cannot be considered the main aetiological factor for RAU. In contrast to the above studies, Lalla et al. found no beneficiary effect from multivitamin therapy on the number and duration of RAU episodes (Lalla et al., 2012). Such results can give an indication that vitamin replacement therapy might be helpful for those RAU patients suffering from vitamin deficiency.

Stress and psychological profiles

Stress has always been regarded as one of the primary predisposing factors for RAU, particularly among laypersons (Zadik et al., 2012).

RAU patients exhibit higher levels of psychological stress and anxiety, and appear angrier than healthy individuals (Albanidou-Farmaki et al., 2008; Al-Omiri et al., 2012; Gallo Cde et al., 2009). A study by Huling et al. concluded that stressful events may be associated with the onset of RAU, but not with the duration of an ulcer (Huling et al., 2012).

In contrast to the above studies, other studies found no differences in the levels of depression and stress between the acute and remission phases of RAU, the periods during which an ulcer was present and absent, or between RAU patients and healthy controls (Huling et al., 2012; Pedersen, 1989).

Decreasing levels of stress positively affected RAU patients. Among 12 RAU patients, symptoms improved after using Flupentixol® and Melitracen® prescribed as antidepressants (Yaacob and Ab Hamid, 1985). The frequency of RAU episodes

significantly decreased after subjecting patients to relaxation and imagery training (Andrews and Hall, 1990).

No clear relationship between RAU and stress has been established and no clear explanation about the role of stress in the onsest of RAU has been found; yet, some scholars suggest that stress may change the immune system of the oral mucosa. Such contradictory results leave the door open regarding the role of stress in RAU.

Smoking and the role of nicotine in RAU

Smoking is regarded as the leading cause of several diseases, including cancer, cardiovascular disease and lung disease among others. Despite its harmful effects on human health, nicotine carries some beneficial effects for RAU patients.

The protective role of smoking against RAU was first reported in 1960 by Bookman, who documented the complete remission of RAU in four patients after the resumption of smoking (Bookman, 1960). Four years later, Drosey reported similar results in three patients (DORSEY, 1964).

A significant negative correlation was found between RAU and smoking in three different studies among three different populations (Axell and Henricsson, 1985; Rivera-Hidalgo et al., 2004; Tuzun et al., 2000). In contrast to these studies, among 1000 students at the University of Jordan, no significant difference in the annual prevalence of RAU was found between smoking and non-smoking students. In the same study, students classified as long-term heavy smokers reported significantly fewer RAU episodes than those identified as moderate smokers for a shorter time (Sawair, 2010).

Individuals who ceased smoking experienced a significant increase in RAU especially after the first week of smoking cessation (Marakoglu et al., 2007; Ussher et al., 2003).

To confirm the protective role of nicotine in RAU, nicotine replacement (nicotine lozenges) were successfully used to treat RAU (Hill et al., 2010).

This protective effect of smoking in general and specifically nicotine was associated with two main factors. First, the increase in keratin production from oral epithelial cells resulted nicotine stimulation; second, the inhibition of the production of pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6, and the increase in the production of anti-inflammatory cytokine IL-10 (Subramanyam, 2011) were associated with nicotine.

Oxidant and antioxidant status in RAU

The total oxidant/antioxidant ratio and total oxidative stress may play important roles in the pathogenesis of RAU.

The total antioxidant status of sera significantly decreases in RAU patients compared with healthy controls, a finding that contradicts the oxidant status and total oxidative stress (Akoglu et al., 2013; Bilgili et al., 2013). A similar pattern could not be found in the saliva, since no difference was found in the total antioxidant capacity, total oxidant status, and the oxidative stress level between RAU and healthy control subjects (Caglayan et al., 2008).

A significant down-regulation in the activity of antioxidant enzymes, such as catalase, glutathione peroxidase, and paraoxonase 1 arylesterase, was found in the plasma, serum, and erythrocytes of RAU patients compared with healthy controls (Arikan et al., 2009; Bilgili et al., 2013; Cimen et al., 2003; Karincaoglu et al., 2005; Ozturk et al., 2013).

Malondialdehyde, the end product of lipid peroxidation and an indicator of oxidative stress, significantly increases in the plasma, serum, and saliva of RAU patients compared with healthy controls (Arikan et al., 2009; Cimen et al., 2003; Saral et al., 2005).

Karincaoglu et al. studied oxidative stress by evaluating the antioxidant enzymes in the plasma and saliva of 30 RAU patients compared with 30 healthy controls. Interestingly, the authors found that the levels of catalase and superoxide dismutase

were significantly lower, while the level of glutathione peroxidase was higher in the plasma of RAU patients compared with healthy controls. The exact opposite was found in the saliva of the same patients and controls from that same study. The authors suggested that this high level of antioxidant enzymes in the saliva may play an important role in protecting the ulcer area. They also argued that the incongruity in the level of antioxidant enzymes between the plasma and saliva may relate to the body's ability to mobilise antioxidant enzymes to the site where they are needed (Karincaoglu et al., 2005).

2.5. Aetiological factors of RAU

Table 2 provides a summary of the aetiological factors associated with RAU (page 14).

Irritating agents

Sodium lauryl sulphate (SLS) is the most common irritant to the oral mucosa given its frequent use in dentifrices. SLS significantly increased the number of RAU episodes, the duration of an ulcer, and the mean pain score (Chahine et al., 1997; Herlofson and Barkvoll, 1994; Shim et al., 2012). The effects of SLS may result from a direct irritation to the oral epithelium (van der Valk et al., 1984; Willis et al., 1989), resulting in sloughing, ulceration, and finally inflammation (Herlofson and Barkvoll, 1993; Rubright et al., 1978; Searls and Berg, 1986); a reduction in the protective layer functioning (Siegel and Gordon, 1985; Siegel and Gordon, 1985); a reduction in the keratinisation index (Jacoby et al., 1975); and the denaturation of proteins due to the high affinity of SLS to proteins (Tzutzumi et al., 1982). Interstingly, Neppelberg et al. (2007) showed that the effects of SLS on human oral keratinocytes depend on the SLS concentration. Thus, a low concentration of SLS (0.015%) led to an increase in the epithelium thickness, the proliferation of epithelial cells, and the expression of E-cadherin, and a very low number of apoptotic cell deaths. By contrast, a high concentration of SLS (>0.15%)

led to a decrease in the epithelium thickness, the proliferation of epithelial cells, the expression of E-cadherin, the detachement of cells from each other, and an increase in epithelial cell deaths (Neppelberg et al., 2007).

Food products and hypersensitivities

Findings suggest that several food products initiate RAU. A significant increase in cow's milk antibodies was found among RAU patients compared with healthy controls, but this difference disappeared when compared with other oral mucosal diseases (Thomas et al., 1973). Gluten sensitivity enteropathy was found just in 2.83% of RAU patients (Shakeri et al., 2009). Other products such as cheese, nuts, chocolate, azo dyes, flavoring agents, and preservatives, may also contribute to RAU (Wright et al., 1986). In contrast, Tarakji et al. found that dietary habits did not affect RAU (Tarakji et al., 2012).

Trauma

Despite assumptions regarding trauma's role in RAU (Wray et al., 1981), few trauma studies exist most likely due to ethical and methodological complexities of such research.

Wray et al. found that mechanical injury may induce RAU in susceptible persons, but not in healthy controls (Wray et al., 1981). The delivery of traumatic epithelium may spur the onset of RAU (Aminabadi, 2008).

Bacterial agents

Several studies have examined the aetiological factors or onset of RAU associated with several types of bacteria. Among these bacteria, *Helicobacter pylori* represented the most intensively studied agent due to its role in gastrointestinal ulcers. The majority of studies failed to find a relationship between *Helicobacter*

pylori and RAU, neither in adults (Chapman et al., 1998; Mansour-Ghanaei et al., 2005; Porter et al., 1997; Shimoyama et al., 2000; Victoria et al., 2003) nor in children (Fritscher et al., 2004). Only one study found that 23 of 32 (71.8%) RAU biopsies tested positive for *Helicobacter pylori,* although this study did not include biopsies from healthy controls (Birek et al., 1999).

While most studies found no relationship between *Helicobacter pylori* and RAU, eradicating the bacterium in RAU patients significantly improved patients' wellbeing vis-à-vis decreasing RAU episodes and alleviating the intensity of symptoms (Albanidou-Farmaki et al., 2005; Karaca et al., 2008). Such effects could, however, result from an increase in vitamin B12 serum (Tas et al., 2013).

Some studies indicated that other bacteria, such as *Streptococcus oralis*, *Streptococcus sanguis strain 2A,* and *Streptococcus pyogenes strain M5*, play a role in the development of RAU (BARILE et al., 1963; Donatsky, 1975; Donatsky, 1976; Donatsky, 1976). Other later studies, however, found no such relationship to RAU for any of these bacteria (Hoover et al., 1986; Riggio et al., 2000).

Marchini et al. studied the oral microbiota of RAU patients and healthy controls, finding that the microbiota greatly differs between these two groups (Marchini et al., 2007).

In addition to the above, from a clinical viewpoint, bacteria are most probably not the main cause for RAU as RAU is not a contagious disease. Furthermore, RAU cannot be treated or prevented by systematic antibiotics.

Viral agents

While some scholars suggest that viruses represent causative agents for RAU, no single virus apears to precipitate RAU. As with the above causative factors, many studies in this field report conflicting results.

Lin et al. studied the presence of five viruses in RAU biopsy samples from 60 patients and 72 biopsy samples from normal mucosa, finding no viruses in the

healthy mucosa. However, the following viruses (with the prevalence provided as percentages) were found in the RAU biopsies: human herpes virus (HHV-8), 26.7%; human cytomegalovirus (HCMV) ,18.3%; Epstein–Barr virus (EBV), 15%; human papilloma virus (HPV), 13.3%, HSV-1, 6.7%, while HSV-2 not found (Lin et al., 2005).

Brice et al. suggested that the presence of HHV-6 in the oral mucosa and peripheral blood of RAU patients may result from normal viral shedding rather than a direct cause of disease (Brice et al., 2000).

Sun et al. put forth another hypothesis about the role of viruses in RAU, hypothesising that EBV-infected epithelial cells are lysed by T-lymphocytes, leading to ulcer formation (Sun et al., 1998).

HCMV was found only in the pre-ulcerative lesions of RAU and not in healthy controls (Sun et al., 1996); no significant difference in the prevalence of HCMV IgG antibodies was detected between RAU patients and healthy participants (Ghodratnama et al., 1999).

In addition, Pedersen et al. failed to cultivate varicella zoster virus (VZV) from a RAU lesion, finding instead viral DNA in RAU samples (Pedersen et al., 1993). That same year and in a different study, Pederson et al. found that the reactivation of VZV and HCMV may be associated with the recurrence of RAU (Pedersen and Hornsleth, 1993).

Measles antigens were detected in RAU samples using immunohistochemistry and they might play a role in down-regulation of CD46 (Czerniniski et al., 2000).

Adenovirus was isolated from herpetiform RAU (Sallay et al., 1973).

Table 2. Aetiological and predisposing factors of RAU.

Predisposing factors	Aetiological factors
Age and sex	Irritating agents
Heredity	Food products and hypersensitivity
Hormonal disturbance	Trauma
Haematological disturbance	Bacterial agents
Stress and psychological profile	Viral agents
Ceasing smoking	
Oxidative stress	

2.6. Behçet's disease

RAU may be associated with several systemic diseases as summarized in Table 3. Behçet's disease represents the most important such disease linked to RAU. Behçet's disease is a chronic inflammatory multisystem disease characterised by recurrent ulceration of the oral and genital mucosa which may involve the eyes (Sakane et al., 1999). In addition, Behçet's disease may affect any of the following systems: vascular, musculoskeletal, gastrointestinal, genitourinary, and nervous (Davatchi et al., 2010; Talarico et al., 2010). Behçet's disease has a particularly high prevalence in those countries situated along what is known as the Silk Road compared with other countries in Northern Europe and North America (Skef et al., 2015). As with RAU, the cause of Behçet's disease remains unknown; however, immune dysregulation may play an important role in disease onset (Evereklioglu, 2005). In addition to immune dysregulation, genetic abnormalities highly associate with Behçet's disease. Several research groups reported a strong association between Behçet's disease and the HLA-B51 molecule (Hughes et al., 2013; Kirino et al., 2013; Mizuki et al., 2000; Ohno et al., 1982; Remmers et al., 2010). Furthermore, Behçet's disease is associated with IL10 and IL23R–L12RB2 in the Turkish population, and across the Middle East, Asia, and Europe (Mizuki et al., 2000; Remmers et al., 2010).

Oral mucosa is the most common site involved in Behçet's disease (>90%) and is characterised by oral ulcerations, which cannot be differentiated from RAU clinically or histopathologically. To determine a Behçet's disease diagnosis, patients must experience at least three episodes of RAU over a 12-month period associated with at least two of the following manifistations: recurrent genital ulceration, eye involvement, or positive pathergy test.

The management of Behçet's disease differs depending on the affected site and the severity of the disease. The eye represents the most complicated site (since it may lead to blindness) followed by nervous system. Most drugs used to treat Behçet's

disease, such as as azathioprine, infliximab, and ethanercept, address the immune system (Melikoglu et al., 2005; Sfikakis et al., 2004; Yazici et al., 1999).

RAU and the oral ulcerations associated with Behçet's disease not only share similarities in their clinical features, but also in the expression of cytokines in the ulcer area given that some of the T-helper (Th)1 cytokines and chemokine receptors increase in both RAU and Behçet's disease oral mucosa. The difference lies in the Th2 cytokine IL-4, which increases only for Behçet's disease but not in RAU (Dalghous et al., 2006).

Table 3. Systemic diseases associated with RAU.

Systemic diseases associated with RAU
Behçet's disease
MAGIC syndrome*
Sweet's syndrome
Cyclic neutropenia
Benign familial neutropenia
PFAPA syndrome**
HIV
Nutritional deficiencies
Inflammatory bowel disease
Reiter's disease
Ulcus vulvae acutum

* Mouth and genital ulcers with inflamed cartilage syndrome
** Periodic fever with aphthae, pharyngitis and adenitis syndrome

2.7. Oral mucosa

Oral mucosa comprises primarily epithelium and lamina propria and is separated from the underlying bone and muscular layer by the submucosa (Figure 2).

Depending on the location and function, three types of oral mucosa exist: the lining, masticatory, and specialised mucosa.

The lining mucosa is covered by non-keratinised stratified squamous epithelium. The connective tissue for this type is more flexible and elastic than masticatory mucosa. The lining mucosa covers the lips, cheek, vestibule, free gingiva, and the soft palate, and is most commonly affected by minor RAU.

The masticatory mucosa is covered by keratinised stratified squamous epithelium to protect the underlying tissue from mechanical trauma during mastication. The masticatory mucosa covers the attached gingiva and the hard palate.

Specialised mucosa represents the third type and covers the tongue.

The superfacial layer of the oral mucosa is composed of stratified squamous epithelium. The epithelium comprises four layers: stratum basale (basal layer), stratum spinosum (prickle layer), stratum granulosum (granular layer), and stratum corneum (keratinised layer) in keratinized epithelium and basal layer, prickle cell layer, intermediate layer and superficial layer in nonkeratinized epithelium. Oral epithelial cells represent 90% of the oral epithelium cells, while melanocytes, Langerhans cells, and some inflammatory cells represent 10%.

While the epithelium protects the underlying tissue from external irritation and microbial invasion, the lamina propria supports the oral mucosa and provides nutrients to the epithelial cells. Several types of cells are present in the lamina propria, including fibroblast, endothelial cells, mast cells, macrophages, and other immune cells such as lymphocytes and plasma cells. All of these cells are immersed in an amorphous ground substance.

The submucosa shares similar features with the lamina propria, but differs in its loose fatty tissue, glandular cells, major blood vessels, and nerves which supply the oral mucosa. In some areas of the hard palate and attached gingiva, submucosa disappear and the oral mucosa is directly attached to the underlying bone.

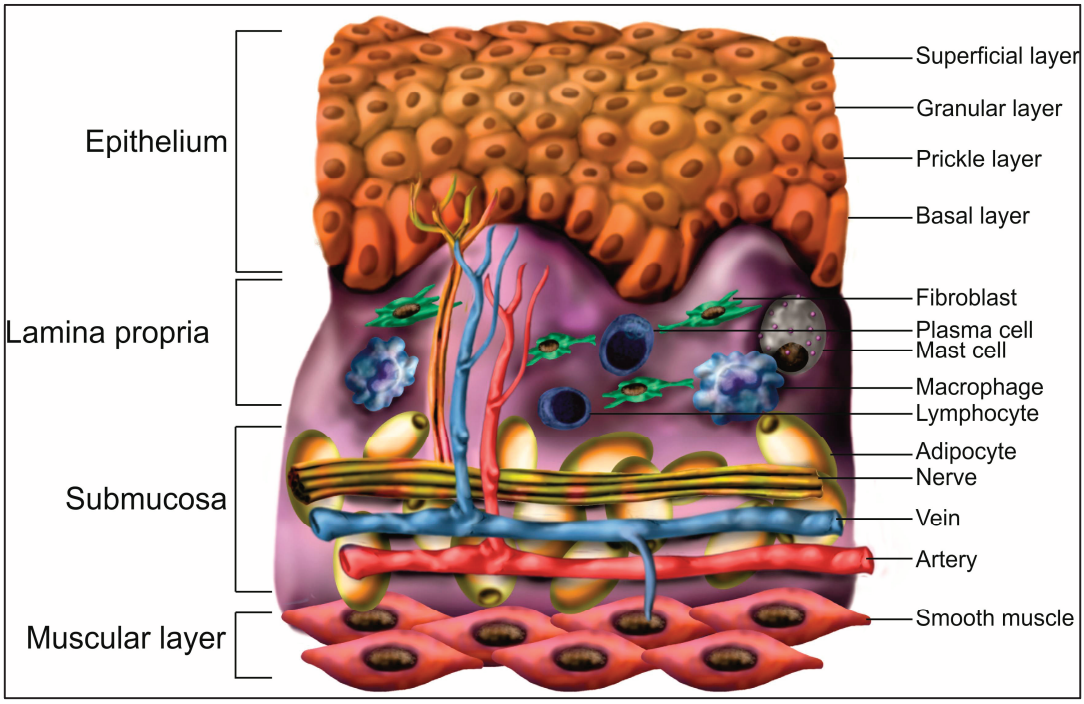

Figure 2. Histological features of oral mucosa.

2.8. Inflammatory cells in RAU

Neutrophils

Neutrophils represent the most abundant white blood cells making up 40% to 70% of all white blood cells. Neutrophils serve as essential elements of innate immunity since these cells are the first to arrive at an inflammation site. Neutrophil cytoplasm contains granules filled with premade microbicidal substances, such as proteolytic enzymes ready for release to attack microbes.

Neturophils highly infiltrate the RAU ulcer area, participating not just in killing the microbes, but also in destroying the surrounding tissue through the release of proteolytic enzymes (Hayrinen-Immonen et al., 1991; Hayrinen-Immonen et al., 1993; Mills et al., 1980).

Two studies on the spontaneous migration of neutrophils in RAU patients yielded different results. While Dagalis et al. found no difference between RAU patients and controls in terms of neutrophil migration (Dagalis et al., 1987), Sistig et al.

found that spontaneous neutrophil migration significantly decreased in RAU patients compared with healthy controls (Sistig et al., 2001).

Furthermore, the phagocytic activity of neutrophils taken from the peripheral blood and saliva of RAU patients is significantly lower compared with the neutrophils taken from healthy controls (Kumar et al., 2010).

Oxygen radicals produced from neutrophils in RAU patients did not differ from the neutrophils in contorls (Wray and Charon, 1991).

Thus, severe infiltration of neutrophils in the ulcer area might indicate the importance of neutrophils in aggravating the inflammation during RAU episode.

Macrophages

When monocytes leave the blood and enter tissue, they proliferate becoming resident macrophages. Resident macrophages form a group of different types of macrophages determined by their location, with each type having its own name. Macrophages play an important role in both innate and adaptive immunity, but the most important function includes scavenging apoptotic bodies and cellular debris under normal conditions; during inflammation, especially during chronic phases of inflammation, macrophages also engulf microbes and clean the area of dead neutrophils, normally appearing during the acute phase of inflammation.

In RAU, macrophages may appear in the lamina propria representing a high percentage of the total immune cell infiltratation especially during the early stage of RAU (Regezi et al., 1993).

Regezi et al. suggested that the endothelial leukocyte adhesion molecule and intercellular adhesion molecule-1 attract macrophages to the RAU lesion, since both stained strongly in the endothelial cells of early RAU lesions. The staining intensity gradually decreased toward the periphery of the lesion (Regezi et al., 1993).

Mast cells

A mast cell is a tissue-resident immune cell first discovered by Paul Ehrlich in 1878.

Mast cells play a major role in several diseases through the release of inflammatory molecules, such as histamine and TNF-α, which are preformed and preserved in a high concentration in granules.

The number of mast cells significantly increase in RAU lesions comparing traumatic ulcers and healthy controls, representing 2% to 5% of the total number of immune cells in RAU (Hayrinen-Immonen et al., 1991; Natah et al., 1998; Schroeder et al., 1983). Mast cells in RAU show signs of degranulation, which support their importance in RAU lesion (Natah et al., 1998). Mast cells have also been identified as a major source of TNF-α in RAU lesions (Natah et al., 1998).

Lymphocytes

Lymphocytes form an important constituent of the white blood cell count since they are regarded as the basic units of adaptive immunity.

Three major types of lymphocytes exist: T cells, B cells, and natural killer (NK) cells. While T cells are responsible for cell-mediated immunity, B cells are involved in humoral immunity. NK cells primarily participate in innate immunity by killing tumor cells and cells infected with viruses, while also playing a role in adaptive immunity.

In 1980, Mills et al. first suggested the role of lymphocytes in RAU, finding that the preulcerative lesion and margins of the developing ulcer contained lymphocytes (Mills et al., 1980).

Several studies focused on the changes in the pattern of lymphocyte types in the lamina propria of the ulcer area and in the peripheral blood of RAU patients. These studies have not always agreed with one another. In two studies, significantly fewer helper T cells (CD4) were found in the lamina propria and peripheral blood of

RAU patients compared with healthy controls (Pedersen et al., 1991; Savage et al., 1988). But, in a third study, no difference was found in the percentage of CD4 cells between RAU patients and healthy controls (Pedersen et al., 1989).

Cytotoxic T cells (CD8) significantly increased (Pedersen et al., 1989; Pedersen et al., 1992; Savage et al., 1988) or did not differ in the lamina propria and peripheral blood of RAU patients compared with healthy controls (Pedersen et al., 1991; Sistig et al., 2001).

Signficantly fewer B cells (CD19) in the active phase of a RAU lesion were found compared with the remission phase (Sistig et al., 2001). This difference, however, was not found in another study comparing the number of B cells (CD19) in the peripheral blood of RAU patients during an active ulcer episode to those in healthy controls (Bachtiar et al., 1998).

Although no difference was found in the number of NK cells in the peripheral blood of RAU patients compared with healthy controls (Bachtiar et al., 1998), the activity of NK cells extracted from RAU patients was significantly lower than that in healthy controls (Sistig et al., 2001).

These results may indicate that an immunological disturbance, represented by an abnormal pattern of lymphocyte distribution in the peripheral blood or in the lamina propria of ulcer area, may play a role either in the onset or progression of RAU.

2.9. Molecular aspects

Toll-like receptors (TLRs)

TLRs comprise a group of receptors belonging to the pathogen-recognition receptor (PRRs) family and play a major role in innate immunity (Akira and Takeda, 2004). Thus far, 13 TLRs have been identified. The toll receptor was first identified in *Drosophila melanogaster;* the first function identified for TLRs included the development and differentiation of the *Drosophila* embryo (Anderson et al., 1985;

Anderson et al., 1985). In 1996, Hoffmann et al. showed that the activation TLRs lead to the production of the antifungal peptide 'drosomycin' which is important in protecting fruit flies from fungal infections (Hoffmann et al., 1999). This discovery proved that the toll receptor features a dual life: one during the development of an embryo and the second during the life of *Drosophila melanogaster*. In 1991, Gay and Keith showed that the cytoplasmic domain of the toll receptor is similar to the cytoplasmic domain of the mammalian IL-1R (Gay and Keith, 1991), named the toll-IL-1 receptor (TIR) (Medzhitov et al., 1997). Since that time, mammalian TLRs were found. While these receptors share cytoplasmic components with IL-1R, they differ markedly from the extracellular region, and IL-1R features a immunoglobulin-like domain in its extracellular region. Finally, TLR contains leucin-rich repeats (Poltorak et al., 1998).

The discovery of TLRs may be regarded as revolutionary in our understanding of the working mechanisms of innate immune systems. For many years, the question of how our body in general and specifically our inflammatory cells recognise microbes or distinguish non-self-molecules from self-molecules and react against them remained unanswered. Scholars assumed that the innate immune cells reacted against microbes in a non-specific manner, while adaptive immune cells reacted in a specific manner. With the discovery of PRRs, the concept changed along with the realisation that these receptors recognise and bind to highly conserved structures of microbes, such as lipopolysaccharide (LPS), lipoproteins, and nucleic acids, in a specific way. Furthermore, PRRs may also recognise and bind to self-molecules usually preserved inside cells, but are either passively released or actively secreted during pathological conditions in order to alert the immune system. For this reason, these molecules are called alarmins or damage-associated molecular patterns (DAMPs).

TLR classification and signalling pathways

Since the discovery of TLR4 as a receptor for LPS, 10 and 13 TLRs have been identified in humans and mice, respectively. In humans, 9 of 10 TLRs possess known ligands and signaling pathways, while the ligands and signaling pathways for TLR10 remain as yet unknown (Table 4).

TLRs are expressed in most mammalian cells starting from the inflammatory cells, such as macrophages, neutrophils, mast cells, and lymphocytes among others, as well as in non-inflammatory cells such as epithelial cells and fibroblasts (Akira et al., 2006).

Members of the TLR family were divided into two groups depending on the cellular location of the receptor. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are located on the cell membrane and recognise microbial structures located on the external surface of the microbes such as LPS and flagellin. The second group of TLRs include TLR3, TLR7, TLR8, and TLR9, which are located in the cytoplasm and recognise microbial nucleic acids (Kawai and Akira, 2010). Stimulation of TLRs with pathogen-associated molecular patterns (PAMPs), microbe-associated molecular patterns (MAMPs), or DAMPs may activate the cells and lead to the production of chemokines, pro-inflammatory cytokines, and antimicrobial peptides. The majority of TLRs work as homodimers, except TLR2 which creates heterodimer complexes with TLR1, TLR6, or TLR10 (Farhat et al., 2008). These heterodimers recognise different subtypes of lipopeptides. While TLR2/TLR1 heterodimers recognise triacylated lipopeptides, TLR2/TLR6 heteropeptides recognise diacylated lipopeptides (Farhat et al., 2008).

Regarding the signaling pathways, all TLRs except TLR3 and TLR4 work through myeloid differentiation factor 88 (MyD88). In addition to MyD88, TLR4 use TIR domain-containing adapter-inducing interferon-β (TRIF) in its signaling pathway. TLR3 is the only receptor in this family which uses TRIF signaling pathways (Kawai and Akira, 2010).

Table 4. Summary of human TLR cellular localisations, signalling adaptors, examples of TLR ligands, and induced effector cytokines.

Receptor	Cellular localisation	Signalling adaptor	Ligand		Effector cytokines induced
			PAMP	DAMP	
TLR1/2	Cell membrane	TIRAP, MyD88	Triacyl lipopeptides		Inflammatory cytokines (TNF-α, IL-6, etc.)
TLR2	Cell membrane	TIRAP, MyD88	Peptidoglycan *Porphyromonas gingivalis* LPS *Bacteroides fragilis* LPS Lipoteichoic acid Lipoarabinomannan Zymosan	HSPs HMGB1 Uric acid Biglycan Hyaluronan Versican	Inflammatory cytokines (TNF-α, IL-6, etc.)
TLR3	Endosome	TRIF	ssRNA dsRNA	RNA	Inflammatory cytokines (TNF-α, IL-6, etc.), type I IFNs
TLR4	Cell membrane	TIRAP, MyD88, TRAM and TRIF	LPS Mannan Envelope proteins	HSPs HMGB1 Uric acid Biglycan Hyaluronan sulphate Tensacin-C Fibrinogen Fibronecting Surfactant B-defensin	Inflammatory cytokines (TNF-α, IL-6 etc.), type I IFNs
TLR5	Cell membrane	MyD88	Flagellin		Inflammatory cytokines (TNF-α, IL-6, etc.)
TLR6/2	Cell membrane	TIRAP, MyD88	Diacyl lipopeptides Lipoteichoic acid Zymosan		Inflammatory cytokines (TNF-α, IL-6, etc.)
TLR7	Endosome	MyD88	ssRNA	RNA	Inflammatory cytokines (TNF-α, IL-6, etc.), type I IFNs
TLR8	Endosome	MyD88	ssRNA	RNA	Inflammatory cytokines (TNF-α, IL-6, etc.), type I IFNs
TLR9	Endosome	MyD88	dsDNA CPG-DNA	DNA	Inflammatory cytokines (TNF-α, IL-6, etc.), type I IFNs
TLR10	Cell membrane				

Data are taken from (Akira and Takeda, 2004; Akira et al., 2006; Erridge, 2010; Kawai and Akira, 2010; Kono and Rock, 2008; Kumar et al., 2009).

TLRs in oral mucosal diseases

Human oral epithelial cells form a physical barrier against mechanical irritation and microbial invasion. In addition, these cells have 10 TLRs (TLR1–10) forming a second line of defence against microbial invasion (Ali et al., 2008). The expression of TLRs was studied in relation to several oral diseases, such as oral lichen planus, chronic hyperplastic candidiasis, periodontitis, oral epithelial dysplasia, and oral squamous cell carcinoma (Ahmed Haji Omar et al., 2015; Ali et al., 2008; Beklen et al., 2008; Janardhanam et al., 2012; Kotrashetti et al., 2013; Siponen et al., 2012). In these studies, the expression of TLRs did not possess the same pattern being instead disease-dependent. TLR2 and TLR4 mRNA expression in the oral mucosal brush samples from Behçet's disease and RAU patients were compared with healthy controls. Both showed significantly higher concentrations in patients experiencing Behçet's disease relapses, while no difference was found in the RAU samples (Seoudi et al., 2014). Gallo et al reported that TLR2 mRNA expression was significantly elevated in RAU tissue samples while TLR3 and TLR5 mRNA exresson was declined when compared with healthy controls (Gallo et al., 2012). Additionally, the peripheral blood mononuclear cells (PBMCs) of RAU patients differed in their response to TLR ligands compared with healthy control, PBMCs of RAU patients showed a higher response to heat-killed Listeria monocytogenes (HKLM) and a lower response to lipoteichoic acid (LTA; (Borra et al., 2009).

High-mobility group box 1 (HMGB1)

HMGB1, a non-histone nuclear protein present in almost all mammalian cells, features a dual life. One life takes place inside the cell participating in transcription, replication, and cellular differentiation during cell division. The second life resides outside the cell when it is actively secreted or passively released during cell death, which works as an inflammatory mediator. The role of HMGB1 in mediating inflammation was first reported by Wang et al. in 1999, finding that cultured

murine macrophages secrete HMGB1 to the supernatant after stimulation with LPS, TNF-α, and IL-1β in a time-dependent manner. HMGB1 also increased in mice serum after mice were exposed to LPS (Wang et al., 1999). As a result of this discovery, HMGB1 was regarded as a late-stage mediator of inflammation and classified as a DAMP.

HMGB1 is located inside the nucleus in most cells, with the ability to continuously shuttle between the nucleus and the cytoplasm in some cells as neutrophils (Ito et al., 2007). HMGB1 is actively secreted by inflammatory cells such as macrophages, monocytes, NK cells, dendritic cells, endothelial cells, and platelets after activation (Harris et al., 2012). During cell activation, HMGB1 undergoes post-translation modification, that is, phosphorylation and acylation. Some of these changes affect the binding of HMGB1 to DNA and prevent HMGB1 from entering the nucleus again, leading to the accumulation of HMGB1 in the secretory lysosomes before secretion to the extracellular matrix (Ito et al., 2007; Youn and Shin, 2006).

HMGB1 may also be passively released to the extracellular matrix during cell necrosis. Some controversy exists regarding the release of HMGB1 from apoptotic cells, since early studies claimed that HMGB1 is stored inside apoptotic bodies during cell apoptosis. Later studies found that HMGB1 is released in an oxidised form (inactive form) as a result of oxidation by mitochondrial reactive oxidative species (ROS) (Andersson and Tracey, 2011; Kazama et al., 2008).

A significant improvement in our understanding of the effects of extracellular HMGB1 on mammalian cells resulted from a study by Venereau et al. in 2012, when they discovered activity differences between the three redox forms of HMGB1 (Venereau et al., 2012).

While all-thiol HMGB1 works as a recruiting agent for leucocytes, disulfide-HMGB1 stimulates macrophages to produce chemokines such as IL-8 and pro-

inflammatory cytokines such as TNF-α and IL-6. The final redox form is terminally oxidised and regarded as inactive (Venereau et al., 2012).

Differences in the redox form also affect the binding of HMGB1 to its receptors. All-thiol HMGB1 forms a complex with C-X-C motif chemokine 12 (CXCL12; HMGB1-CXCL12 heterocomplex) which later to binds C-X-C chemokine receptor type 4 (CXCR4) and leads to the recruitment of inflammatory cells. After the formation of an intramolecular bond, HMGB1 loses its ability to bind with CXCL12 leading to the inhibition of its chemotaxis effect. Disulfide-HMGB1 binds to TLR4 and activates the nuclear factor kappa-light chain enhancer of activated B cells (NF-κB) through an MyD88-dependent mechanism leading to cell activation and the production of pro-inflammatory cytokines and chemokines (Venereau et al., 2012). In addition to its role in inflammation, HMGB1 in association with p53 may regulate the balance between apoptosis and autophagy. p53 knockout colon epithelial HCT116 cells increase the cytosolic HMGB1, which is associated with an increased autophagy and decreased apoptosis. In contrast, HMGB1 knockout cells increase the cytosolic p53, which is associated with an increasing apoptosis and decreasing autophagy (Kristen et al., 2012).

Redox forms also differ depending on the location of HMGB1. While in the resting state, HMGB1 remained inside the cells usually in the all-thiol form; that which is secreted may appear in any of the three forms. It was also reported that in acetaminophen-induced liver injury, the chemotactic all-thiol HMGB1 was elevated first in mice serum leading to the recruitment of inflammatory cells. This is regarded as the first step of inflammation, followed by an increase in the level of inflammatory mediator disulfide-HMGB1 in the serum. When the liver inflammation resolved, the C106 terminally oxidised HMGB1 predominated in mice serum (Antoine et al., 2010; Antoine et al., 2012; Yang et al., 2013).

HMGB1 in oral mucosal diseases

As HMGB1 is a relatively newly discovered inflammatory mediator, its function in oral diseases remains insufficiently studied. In this context most of the papers have been focused on HMGB1 role in in periodontitis. Ligature- or LPS-induced periodontitis in rats have been associated with the increase in the HMGB1 mRNA (Nogueira et al., 2014). In human chronic periodontitis, HMGB1 mRNA significantly increased when compared with control gingival tissue (Luo et al., 2011). This increase was also associated with the translocation of HMGB1 from the nucleus in healthy gingival epithelial cells to the cytoplasm in periodontitis gingival epithelial cells (Ebe et al., 2011).

Interleukin-17C (IL-17C)

IL-17C was discovered in 2000 by Li et al. (Li et al., 2000). IL-17C was classified as a member of the IL-17 family, a group of cytokines that play an important role in the human immune system through the activation of mammalian cells to produce pro-inflammatory cytokines, chemokines, and antimicrobial peptides (Table 5). IL-17A was the first member of this family identified, which then expanded to inlcude five additional members to form a family. Six cytokines work through five different receptors, which include IL-17RA–RE (Pappu et al., 2012). IL-17A and IL-17F are the best-known cytokines from this family, and have been studied intensively in relation to several autoimmune and inflammatory diseases, primarily rheumatoid arthritis and psoriasis (Iwakura et al., 2011). Other members of this family have clearly received less attention. Studying the effect of IL-17C and its active mechanisms largely began after the discovery that epithelial cells represent the primary producers of this cytokine (Ramirez-Carrozzi et al., 2011).

IL-17C exerts its effect through binding to the IL-17RA and IL-17RE heterodimers, where the affinity to bind to IL-17RE is higher than that in IL-17RA (Ramirez-Carrozzi et al., 2011). However, both are needed to stimulate cells.

Scholars found that blocking IL-17RA in human primary keratinocytes led to an inhibition in the production of granulocyte colony-stimulating factor (G-CSF) and beta defensin (BD)-2 in a concentration-dependent manner. Similarly, IL-17RE as fibroblasts failed to respond to IL-17C despite having IL-17RA while lacking IL-17RE (Ramirez-Carrozzi et al., 2011).

The importance of IL-17C was identified when the TLR2 and TLR5 ligands were found to stimulate the epithelial cells to produce IL-17C. This effect was not found for other IL-17 family members. In addition to PAMPs, pro-inflammatory cytokines such as TNF-α and IL-1β also activate epithelial cells to produce IL-17C (Ramirez-Carrozzi et al., 2011).

In vivo and *in vitro* studies showed that IL-17C carries both beneficial and detrimental effects. IL-17C and IL-17RE knockout mice suffered severe weight loss and a huge bacterial burden, and none survived after challenged by bacteria (Reynolds et al., 2012). The stimulation of epithelial cells with IL-17C led to a significant increase in the production of antimicrobial peptides (Ramirez-Carrozzi et al., 2011). This evidence clearly indicate that IL-17C is quite important in the defence against microbial challenges. For this reason, the idea that IL-17C may provide sufficient defence in the epithelium tissue during weak microbial challenges was put forth, without recruiting inflammatory cells and without producing pro-inflammatory cytokines.

However, this was simply identified as the first phase of IL-17C; the second phase appears as a severe inflammation. In such cases, IL-17C stimulates human cells, especially epithelial cells since few have IL-17RE. This results in the production of pro-inflammatory cytokines and chemokines, which later work to recruit inflammatory cells ultimately leading to severe inflammation and the destruction of the tissue.

Table 5. Summary of human IL-17 family members, receptors, and their producing cells.

IL-17 member	IL-17 receptor	Producing cells
IL-17A	IL-17RA/IL-17RC	$CD4^+$ T cell, $CD8^+$ T cell, γδ, T cell, NKT cell, neutrophil and paneth cell.
IL-17B	IL-17RB	Chondrocyte, neuron, cells of the gastrointestinal tract, and pancreas.
IL-17C	IL-17RE/IL-17RA	Keratinocyte, $CD4^+$ T cell, dendritic cells, and Macrophage.
IL-17D	unknown	$CD4^+$ T cell and B cell
IL-17E	IL-17RA/IL-17RB	$CD4^+$ cell, $CD8^+$ T cell, mast, cell, eosinophil, epithelial cell and endothelial cell
IL-17F	IL-17RA/IL-17RC	$CD4^+$ cell, $CD8^+$ T cell, γδ, T cell, NKT cell, LTi-like cell, and epithelial cell

Modified from Iwakura et al (Iwakura et al., 2011).

IL-17C in oral mucosal diseases

While IL-17C plays a major role in the regulation of the epithelial immunity (Ramirez-Carrozzi et al., 2011), its role in oral diseases has received insufficient attention. IL-17C and its receptor IL-17RE have not been found of importance in the immunity against oral and cutaneous Candida infections (Conti et al., 2015). Because IL-17A received primary attention in comparisons with other IL-17 family cytokines, this was also the case in oral mucosal diseases. IL-17A is overexpressed in oral lichen planus and chronic periodontitis compared with healthy controls (Lu et al., 2014; Mitani et al., 2015). In addition, patients with a IL-17A production defect or IL-17A autoantibodies suffer from chronic mucocutaneous candidiasis (Conti et al., 2011; Kisand et al., 2010). The IL-17A defence mechanism against *Candida* infection is primarily dependent on the induction of antimicrobial peptides (AMPs; (Trautwein-Weidner et al., 2015).

Beta 2 defensin (BD-2)

Defensins form a group of endogenous cystin-rich AMPs, regarded as the largest group of the AMPs family (Dale and Krisanaprakornkit, 2001). Defensins are cationic peptides with a molecular mass of 3.5 to 4.5 KD (Bals, 2000). Defensins possess two terminals: an amino terminal which works mainly against gram positive bacteria and a carboxyl terminal which works against gram negative bacteria (Zhu and Gao, 2013).

Defensins are divided into three main groups: α, β, and θ (the newest member of the family) (Pazgier et al., 2006). Each of these three groups has their own family. BD is the most important member of the defensin family given that the mucosa is produced primarily from epithelial cells and is regarded as a part of the first-line defence against microbial invasion (Bals, 2000).

Several forms of BDs were identified, while the first three (BD-1, -2, and -3) represent the most well-known, more intensively studied members. Because BD-1 is constantly expressed by the epithelial cells in healthy and in inflamed tissue, it is regarded as a static defence mechanism. BD-2 and BD-3 are secreted only after activation of the epithelial cells and are expressed primarily in inflamed tissue and sometimes at a very low level in healthy tissue (Gursoy and Kononen, 2012).

BD-2 is an inducible AMP which works primarily against gram negative bacteria. BD-2 was first identified in humans in 1997 when it was purified from the skin of psoriasis patients (Harder et al., 1997). Several agents may induce BD-2 production. These agents may be divided into microbial products as gram negative bacteria, candida, and others; and pro-inflammatory cytokines may include interferon gamma (IFN-γ), TNF-α, IL-1β, IL-6, IL-17, and IL-22 (Kanda et al., 2011). Both NF-κB and activator protein 1 (AP-1) play a major role in the induction of BD-2 since it shares several binding sites with them (Fellermann and Stange, 2001).

BD-2 has several functions. It is primarily known as a killing tool against microbes by attacking the microbial membrane and forming multimeric pores (Bals, 2000). Surprisingly, studies showed that BD-2 may accomplish more than simply killing microbes since it was found to work as a chemotaxis agent for neutrophils, mast cells, and other inflammatory cells (Diamond and Ryan, 2011). In addition, BD-2 helps in the healing of wounds through increasing the migration of keratinocytes. This may explain the significant increase in BD-2 expression at the wound edge. Such an increase in this expression may result from the synergistic effect of the epidermal growth factor (EGF) and IL-1α (Diamond and Ryan, 2011).

Since the identification of the first AMP, many researchers assumed that these peptides could serve as new antibiotics. By using them, we may overcome the problem of microbial resistance, which is regarded as a serious problem especially as the number of such microbes continuously increases. Unfortunately the use of AMPs was not so successful and most of them were failed to pass the clinical trials mainly because of the high cost and also because no significant difference was found between AMPs and synthetic antibiotics. Despite these unsatisfying results for some AMPs, clinical trials for other AMPs continue. Magainin peptide, which is now in the third phase of clinical trials for the treatment of diabetic foot ulcers, may represent the first AMP released on the market (Fox, 2013; Gordon et al., 2005).

BD-2 in oral mucosal diseases

BD-2 is an inducible AMP expressed only in inflamed tissue (Gursoy and Kononen, 2012). BD-2 mRNA increases in the oral mucosa of gingivitis and periodontitis compared with control oral mucosa (Dommisch et al., 2005). At the protein level, BD-2 is found to be increased in *Candida* infected oral mucosa of patients with recurrent respiratory papilomatosis (Chong et al., 2006; Sawaki et al., 2002). Additionally, salivary BD-2 level have been increased in patients with oral

lichen planus, Behçet's disease, and RAU compared with healthy controls and returns to a normal level after treatment (Kucukkolbasi et al., 2013).

3. Aims of the study

The principal aim of this study was to identify the most important steps in the initiation and down-regulation of the inflammation in RAU lesions and the role of oral epithelial cells in this process.

In order to achieve the principal aim of this study, we focused on addressing four specific aims:

A) To study the mechanism of oral epithelial cell death in RAU and the effects of this on the adjacent cells.

B) To study the pattern of TLR expression in RAU lesions and compare this expression to that in healthy controls.

C) To study the expression of selected chemokines, pro-inflammatory cytokines, and antimicrobial peptides in RAU lesions and compare this expression to that in healthy controls.

D) To study the response of oral keratinocytes to pro-inflammatory cytokines in terms of TLR up-regulation and in terms of chemokine, pro-inflammatory cytokine, and antimicrobial peptide production.

4. Patients and methods

4.1. Patients

We selected 13 subjects suffering aphtae and 11 healthy controls for inclusion in this study. We took biopsy samples from the aphtae lesions from 8 men and 5 women (mean age of 33.7 years, range of 10 to 63 years). The lesions were taken from labial and buccal mucosa, with the ulcer duration ranging from 4 to 7 days. We only included minor RAUs in this study, taking biopsies only from ulcers which fulfilled the criteria of minor RAU, which were round or oval in shape, measuring less than 10 mm in diameter, appearing yellow or gray in colour, recurring, and surrounded by a red erythematous halo area.

We took control samples from healthy oral mucosa from 4 men and 7 women (mean age of 31.2 years, range of 12 to 67 years) during wisdom and canine tooth extraction. A specialised oral surgeon performed all biopsies under local anesthesia (xylocaine-adrenaline, 20 mg/ml 12.5 μg/ml). Samples were preserved in 10% formalin and then embedded in paraffin blocks according to a standard protocol. The study protocol was approved by the ethics committee of the Institute of Dentistry, University of Helsinki (Dnro 109/13/03/02/2012). Patient participation was voluntary and all patients signed an informed consent form.

To avoid secondary RAU, all patients suffering from Behçet's disease, periodic fever, cyclic neutropenia, Reiter's syndrome, Sweet syndrome, an immunodeficiency such as human immunodeficiency virus (HIV), or recent viral infections were excluded from the study. Patients receiving topical or systemic treatment with corticosteroids or taking any medications that affect the RAU cycle were also excluded from the study.

Neither patients nor controls had an iron, vitamin B12, or folic acid deficiency, and none were using drugs known to induce aphthae, such as methotrexate.

4.2. Methods

4.2.1. Cell culture

Human oral primary keratinocytes (HOK)

Human oral primary keratinocyte cells and keratinocyte culture medium were purchased from Science Cell Research Laboratory (Science Cell Research Laboratory, Carlsbad, CA, USA). The cell culture medium was supplemented with oral keratinocyte growth supplement and a 500 unit/ml of penicillin/streptomycin solution. Cells were cultured in a 75 T-flask until 80% confluent was achieved. For stimulation, cells were harvested by adding trypsin-ethylenediaminetetraacetic acid (EDTA), and were then seeded in a 24-well plate with a density of 5 x 10^4 cells/well. The cells were exposure to different human proteins as outlined in Table 6.

Human oral keratinocyte SCC-25 cell line

The SCC-25 (ATCC® CRL-1628™) cell line is derived from tongue cancer and was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in a 10-cm dish supplemented with Dulbecco's Modified Eagle Medium (DMEM)-12 (Gibco, Payisley, UK), 10% fetal bovine serum, 1000 U/ml penicillin, and 0.1 mg/ml streptomycin. At 80% confluence, the cells were detached by trypsin-EDTA and plated in a 24-well plate at 1 x 10^5 cells/well. Table 6 summarises the protein concentrations and the duration of the stimulations.

Human primary gingival fibroblasts

Human primary gingival fibroblasts were isolated using the explant culture technique (Konttinen et al., 1989). Cells were grown in a 10-cm dish containing RPMI-1640 medium (Lonza, Basel, Switzerland) with 10% fetal bovine serum, L-glutamine, 1000 U/ml penicillin, and 0.1 mg/ml streptomycin. The cells were detached with trypsin-EDTA and seeded in a 12-well plate at a density of 8 x 10^4

cells as decribed above. Table 5 summarises the protein concentrations and the duration of the stimulations. Table 6 describes the cell exposure to different human proteins.

Table 6. Details of the protein concentrations and duration of stimulations used in this study.

Protein	Concentration	Duration (hours)	Company	Study
All-thiol-HMGB1	1000 ng/ml	8, 24	R&D system	I
Di-sulfide-HMGB1	1000 ng/ml	8, 24	R&D system	I
IL-17C	100 ng/ml	8, 24	R&D system[1]	III
		24		IV
IFN-γ	50 ng/ml	8, 24	R&D system	I
LPS	10 ng/ml	24	R&D system	I
Self-DNA	50 ng/ml	8, 24	Extracted from SCC-25 cells	I
TNF-α	10 ng/ml	4	R&D system	I
	50 ng/ml	24		IV
Vitamin K3	10 µM	24	Sigma[2]	IV

1) R&D system, Minneapolis, MN, USA.
2) Sigma, St. Louis, MO, USA.

4.2.2. Immunohistochemistry

Formalin fixed and paraffin embedded tissue samples were cut to a 4-µm thickness and incubated overnight at 37°C. For the first study, the slides were stained with an automated robot machine (Leica BOND-MAX, Leica Microsystems, Wetzlar, Germany). The machine was supplied with a bond-polymer refined detection kit for staining and a citrate buffer solution for antigen retrieval. According to the selected protocol, the slides were incubated for 20 min in the citrate buffer, 60 min in the primary antibody, 30 min in the post primary, 30 min in the polymer, 10 min in 0.023% 3,3'-diaminobenzidine tetrahydrochloride (DAB), and 5 min in hematoxylin. The slides were washed with a washing buffer (supplied from the company) between steps.

For the other three studies, a manual staining protocol from VECTASTAIN ABC Systems (Vector Laboratories, Burlingame, CA) was used. The slides were first deparaffinised, and antigens were retrieved in a citrate buffer (pH 6.0) under a microwave for 25 min (MicroMED T/T Mega Histoprocessing Labstation; Milestone Srl, Sorisole, Italy). Endogenous peroxide was blocked using 3% H_2O_2 in a phosphate-buffered saline (PBS) for 10 min. A nonspecific background staining was blocked by incubating the slides with a 10% normal serum for 1 h at room temperature (RT). Then, the slides were incubated with primary antibodies (Table 7) overnight at +4°C. The next day, the slides were incubated with biotin-conjugated secondary antibody for 1 h at RT. Then, they were incubated with an avidin–biotin complex for 1 h at RT. To develop the colour, 0.006% hydrogen peroxide substrate and DAB chromogen were added to the slides, and the slides were incubated for 10 min at RT. Hematoxylin staining was used to stain the cell nuclei. Between steps, the slides were washed three times for 5 min each with PBS or 0.5% Triton X-100 diluted in PBS.

For negative staining of controls, slides were incubated with a non-immune IgG at the same concentration, the same incubation time, and in the same environment as the primary antigen-specific antibodies.

4.2.3. Immunofluorescence

Paraffin-embedded tissue sections and cultured cells on coverslips were permeabilised with 0.5% Triton X-100 for 10 min before incubating them in a 10% normal serum for 1 h at RT. The slides and coverslips were incubated with primary antibodies overnight at +4°C (Table 7). Then, they were incubated with fluorescein-conjugated secondary antibodies for 1 h at RT. Nuclear staining relied on 4'6-Diamidino-2-phenylindole (DAPI) for 10 min at RT. Finally, samples were mounted in Vectashield® (Vector Laboratories, Burlingame, CA) and kept at +4°C

in a dark place. The slides and coverslips were washed three times with PBS for 5 min each between steps.

For the negative staining of controls, slides were incubated with a non-immune IgG at the same concentration, the same duration, and in the same environment as the primary antigen-specific antibodies.

Table 7. Detailed information about the antibodies used in this study.

Antibody	Type	Company	Concentration	Staining method	Study
4HNE	Polyclonal rabbit IgG	Abcam[1]	2 µg/ml	Immunohistochemistry	IV
BD-2	Polyclonal rabbit IgG	Bioss[2]	2 µg/ml 4 µg/ml	Immunohistochemistry Immunofluorescence	IV IV
Caspase-3	Polyclonal rabbit IgG	Cell Signaling Technology Inc.[3]	0.5 µg/ml	Immunohistochemistry	I
CD68	Monoclonal mouse IgG1	DAKO[4]	1 µg/ml	Immunofluorescence	IV
HMGB1	Monoclonal mouse IgG1	Abnova[5]	1 µg/ml 10 µg/ml	Immunohistochemistry Immunofluorescence	I I
IL-8	Polyclonal goat IgG	Santa Cruz[6]	0.2 µg/ml	Immunohistochemistry	III
IL-17A	Polyclonal goat IgG	R&D	1 µg/mL 5 µg/mL	Immunohistochemistry Immunofluorescence	III III
IL-17C	Polyclonal goat IgG	R&D	1 µg/mL	Immunohistochemistry	III
MCT	Monoclonal mouse IgG1	AbD Serotec[7]	0.1 µg/ml	Immunofluorescence	III, IV
TLR1	Polyclonal rabbit IgG	Santa Cruz Biotechnology	0.8 µg/mL	Immunohistochemistry	II
TLR2	Polyclonal rabbit IgG	Santa Cruz Biotechnology	2.6 µg/mL 2 µg/mL	Immunohistochemistry Immunofluorescence	II I
TLR3	Polyclonal rabbit IgG	Santa Cruz Biotechnology	2 µg/mL	Immunohistochemistry	II
TLR4	Polyclonal rabbit IgG	Santa Cruz Biotechnology	1.3 µg/mL 2 µg/mL	Immunohistochemistry Immunofluorescence	II I
TLR5	Polyclonal rabbit IgG	Santa Cruz Biotechnology	1.3 µg/mL	Immunohistochemistry	II
TLR6	Polyclonal goat IgG	Santa Cruz Biotechnology	1 µg/mL	Immunohistochemistry	II
TLR7	Polyclonal goat IgG	Santa Cruz Biotechnology	0.8 µg/mL	Immunohistochemistry	II
TLR8	Polyclonal rabbit IgG	Santa Cruz Biotechnology	1 µg/mL	Immunohistochemistry	II
TLR9	Polyclonal rabbit IgG	Santa Cruz Biotechnology	0.5 µg/mL	Immunohistochemistry	II
TLR10	Polyclonal goat IgG	Santa Cruz Biotechnology	1.6 µg/mL	Immunohistochemistry	II
TNF-α	Monoclonal mouse IgG1	RDI[8]	0.5 µg/ml	Immunohistochemistry	III

1) Abcam, Cambridge, UK. 2) Bioss, Woburn, MA, USA. 3) Cell Signaling Technology Inc., Danvers, MA, USA. 4) DAKO,Glostrup, Denmark . 5) Abnova, Taipei city, Taiwan. 6) Santa Cruz Biotechnology, Santa Cruz, CA, USA. 7) AbD Serotec, Oxford, UK. 8) RDI, Flanders, Belgium.

4.2.4. TUNEL staining

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was completed using the DeadEnd Colorimetric TUNEL System (Promega Corporation, Fitchburg, WI, USA). Here, 4-µm-thick sections were deparaffinised and incubated in 20 µg/ml proteinase K solution for 30 min at 37°C, an equilibration buffer for 10 min at RT, and a rTdT reaction mix for 60 min at 37°C. The reaction was terminalised by incubating the slides in 2 x SSC for 15 min at RT, and an endogenous peroxide was blocked by 0.3% H2O2. The slides were incubated in streptavidin HRP for 30 min at RT, and then the colour was developed by incubating the slides in DAB substrate for 10 min at RT. The slides were washed three times for 5 min in PBS after each step.

4.2.5. DNA extraction

We extracted DNA from SCC-25 cells using a DNA extraction kit (Macherey-Nagel, Düren, Germany). The DNA concentration was measured using the NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

4.2.6. Quantitative real-time PCR (qPCR)

Cells were lysed using a RLT lysis buffer and RNA was extracted using the RNeasy Mini Kit (Qiagen, Düsseldorf, Germany). The total amount of RNA was measured using the NanoDrop 1000 spectrophotometer (Thermo scientific, Waltham, MA, USA). cDNA was synthesised by adding an equal amount of total RNA to 4 µl of reaction mix and 1 µl of transcriptase enzyme to reach a total volume of 20 µl. The iScript cDNA synthesis kit was used in all studies (Bio-Rad, Hercules, CA, USA).

To run qPCR, each well in the 96-well plate contained 10-µl iQ SYBR green, 7-µl water, 1-µl 250 nM primer, and 2-µl cDNA.

We used two housekeeping genes. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used in the first and third studies, and ribosomal protein large P0 (RPLP0) was used in the first and fourth studies. Table 8 provides the gene sequences of the human primers used in this study.

Table 8. Sequence of the human primers used in this study.

Gene	Forward	Reverse	Study
BD-2	5'-ATCAGCCATGAGGGTCTTGT-3'	5'-GAGACCACAGGTGCCAATTT-3'	IV
GAPDH	5'-AAGGTCATCCCTGAGCTGAA-3'	5'-TGCTGTAGCCAAATTCGTTG-3'	I, III
IL-17A	5'-CTACAACCGATCCACCTCACCTTG-3'	5'-GGTAGTCCACGTTCCCATCAGC-3'	III
IL-17C	5'-CGCTGCCGCCACCATGAC-3'	5'-GCAGTTCCTCAGCCGAGTAGC-3'	III
IL-17RA	5'-CTGGTTCATCACGGGCATCTCC-3'	5'-GGTGGTCGGCTGAGTAGATGATC-3'	III
IL-17RE	5'-TCCTGGAATGTAAGCATGGATACC-3'	5'-GGAAGGGAATGATGAGGTCTAGTG-3'	III
IL-6	5'-AGGAGACTTGCCTGGTGAAA-3'	5'-GAGGTGCCCATGCTACATTT-3'	I
IL-8	5'-TCTGCAGCTCTGTGTGAAGG -3'	5'-ACTTCTCCACAACCCTCTGC-3'	I, III
RPLP0	5'-GGCGACCTGGAAGTCCAACT-3'	5'-CCATCAGCACCACAGCCTTC-3'	I, IV
TNF-α	5'-GACAAGCCTGTAGCCCATGT-3'	5'-TTGATGGCAGAGAGGAGGTT-3'	I, III

4.3. Microscopy and image analysis

Immunostained sections and cells were analysed under a Leica DM6000 B/M light microscope connected to a digital camera (DFC420 and DFC365FX, Leica Microsystems, Wetzlar, Germany).

In order to compare the staining intensity between RAU and control samples, the mean gray values were measured using the Image J program (Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA). A stop filter type was used with a hue value between 0 and 161. The relative staining intensity was determined by applying the inverse mean grey value.

For the immunofluorescence staining, the corrected total cell fluorescence was measured to compare the staining intensity between the control and stimulated cells.

In order to count the number of positive cells in the lamina proparia, we used the point counting method from three randomly selected areas under a high-magnification power field for each slide (400x).

4.4. Statistical analysis

Results are presented as means and standard deviation. The Levene test was used to check the homogeneity of the variances. In order to compare two groups, Mann–Whitney U test was used in the first and fourth studies and student T test was used in the third study. For multiple group comparisons, a one-way ANOVA followed by a Scheffe post hoc test was used in the first study and a one-way ANOVA followed by a Fisher's least significant difference (LSD) post hoc test was used in the fourth study and. A p value of ≤ 0.05 was considered statistically significant and given as * ($P \leq 0.05$), **($P \leq 0.01$), ***($P \leq 0.001$), ****($P \leq 0.0001$).

5. Results

5.1 Expression of the apoptosis marker, danger signals, chemokines, pro-inflammatory cytokines, antimicrobial peptides, and oxidative stress marker in healthy and RAU oral mucosa

Apoptosis marker caspase-3

The caspase-3 staining was negative in the control epithelium and lamina proparia except for a few connective tissue cells undergoing apoptosis during normal tissue regeneration processes. The RAU epithelium stained strongly positive for caspase-3 especially in the spinous layer. The caspase-3 positive cells were also more numerous in the RAU lamina propria (Figure 3). The apoptotic death of oral epithelial cells in RAU were confirmed by TUNEL staining (data not given).

Danger signal HMGB1

The HMGB1 expression pattern was different between the the control and RAU epithelium. In the control epithelium, HMGB1 was expressed only in the basal layer located in the perinuclear area. In RAU, HMGB1 was expressed in all layers of the epithelium located more in the cytoplasm and the extracellular space. The HMGB1 positive cells were more numerous in the lamina propria of RAU lesions compared with the control lamina propria (Figure 3).

IL-17A and IL-17C

Oral epithelial cells were IL-17A negative both in the control and RAU samples (Figure 3). The inability of oral epithelial cells to synthesise IL-17A was confirmed by qPCR (Table 9). Some cells in the lamina propria stained strongly positive for IL-17A. Double immunofluorescence staining of the oral mucosa with IL-17A and

mast cell tryptase (MCT) proved that mast cells serve as the major producer of IL-17A in the oral mucosa (Figure 3).

IL-17C was weakly positive in control epithelial cells. By contrast, in RAU epithelial cells, it stained stained strongly positive (Figure 3). The ability of oral epithelial cells to synthesise IL-17C was confirmed by qPCR (Table 9). The up-regulation of IL-17C in RAU epithelial cells was confirmed by measuring the relative staining intensity, which was significantly higher in RAU epithelial cells compared with healthy control ($p = 0.006$; see Table 10).

Inflammatory leukocytes in the lamina propria and oral epithelium were also IL-17C positive (Figure 3).

Both IL-17RA and IL-17RE mRNA were expreseed in the oral epithelial cells (Table 9).

Table 9. mRNA expression of IL-17A, IL-17C, IL-17RA, and IL-17RE in primary human oral keratinocytes.

Gene	Expression
IL-17A	-
IL-17C	+
IL-17RA	+
IL-17RE	+

(-) mRNA was under a detectable level, (+) mRNA was detectable.

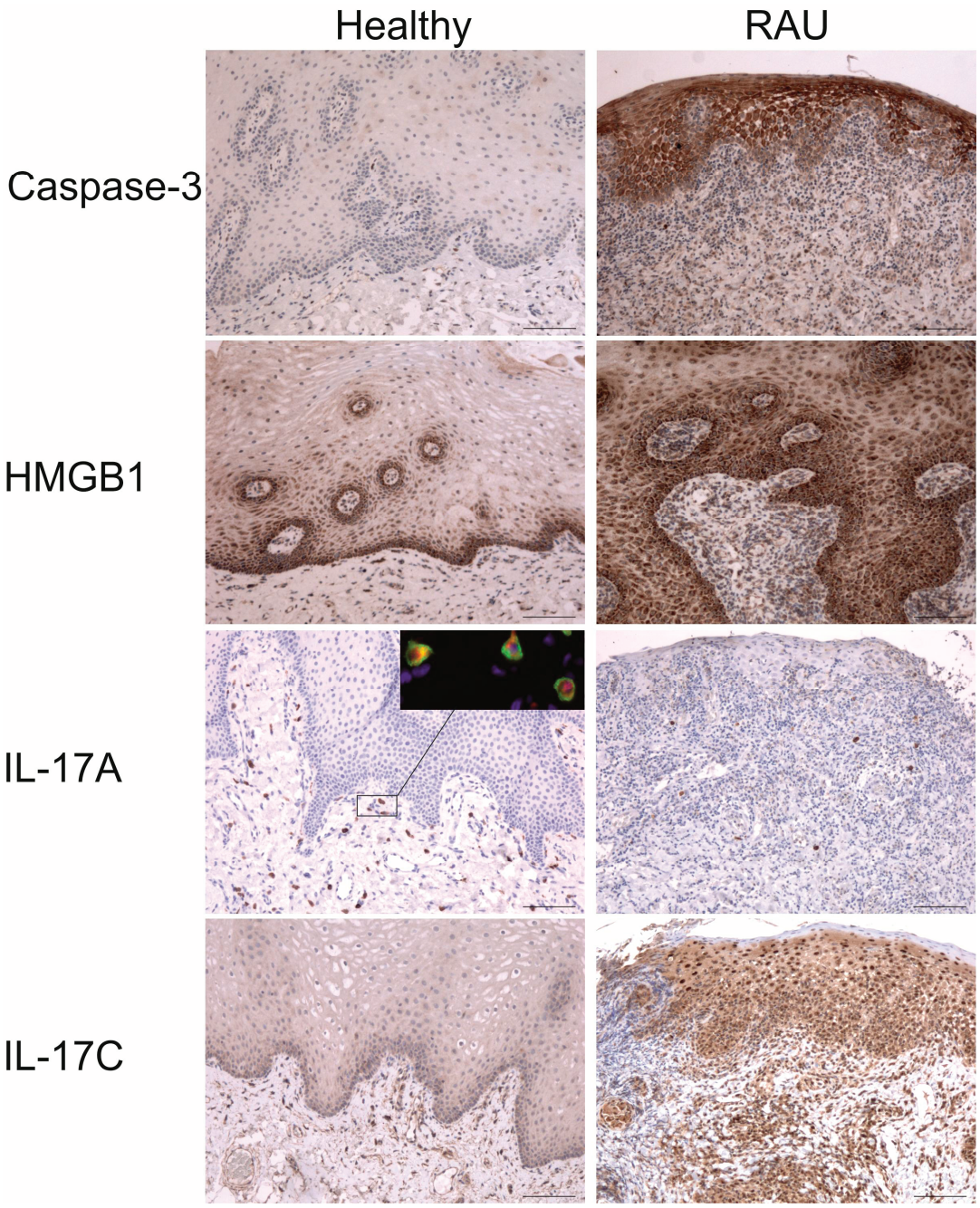

Figure 3. Expression of a select apoptosis marker, a danger signal, and pro-inflammatory cytokines in control and RAU oral mucosa. Control and RAU oral mucosa were immunostained for caspase-3, HMGB1, IL-17A, and IL-17C. Double immunofluorescence staining was completed for MCT and IL-17A: DAPI (blue), IL-17A (red), and MCT (green). Scale bar = 100 μm.

Chemokine IL-8

While oral epithelial cells in the control samples stained either negative or slightly positive for IL-8, RAU epithelial cells were strongly positive with a staining pattern similar across all layers of the epithelium and no difference found between the basal and superficial layers. Some cells in the control and RAU lamina propria stained positive for IL-8 (Figure 4). The relative staining intensity confirmed the up-regulation of IL-8 in RAU epithelium compared with healthy control ($p = 0.02$; see Table 10).

Pro-inflammatory cytokine TNF-α

The oral epithelial cells in the controls stained slightly positive for TNF-α. By contrast, the oral epithelial cells in RAU stained strongly positive. This increase was confirmed by measuring the relative staining intensity ($p = 0.04$; see Table 10). The superficial layer was TNF-α negative in both healthy and RAU epithelium.

Only a few cells in the healthy lamina proparia were positive for TNF-α. In RAU, inflammatory cells infiltrated the lamina proparia and stained positive for both TNF-α (Figure 4).

Antimicrobial peptides BD-2

We found a significant difference in the expression of BD-2 between healthy and RAU mucosa. The control epithelium was either BD-2 negative or weakly positive, with only a few cells in the lamina propria staining positive for BD-2 (Figure 4). RAU epithelial cells stained strongly positive for BD-2 and the number of BD-2-postitive cells in the lamina proparia was significantly higher than that in healthy mucosa ($p = 0.004$; see Table 10).

While mast cells and macrophages were identified as the primary producers of BD-2 in healthy lamina propria, in RAU, BD-2 was primarily produced from infiltrated inflammatory cells (Figure 5).

Oxidative stress marker 4-hydroxynonenal (4HNE)

Both healthy and RAU epithelial cells stained positive for 4HNE. In healthy epithelium, all layers were positive, except for the uppermost layer which was negative. In RAU, staining was stronger, but did not reach statistical significance ($p > 0.05$; see Table 10). In contrast to healthy epithelium, the uppermost layer stained intensely positive for 4HNE, and even stronger than other layers of the RAU epithelium (Figure 4).

Table 10. Staining intensity of select antigens in control and RAU oral epithelium.

Antigen	Staining intensity		p values
	Control	RAU	
IL-17C	0.3 ± 0.09	1.0 ± 0.6	p = 0.006
IL-8	0.8 ± 0.06	1.0 ± 0.2	p = 0.02
TNF-α	0.1 ± 0.1	1.0 ± 1.0	p = 0.04
BD-2	1.0 ± 0.2	5.0 ± 6.5	p = 0.001
4HNE	1.0 ± 1.0	2.2 ± 1.6	p > 0.05

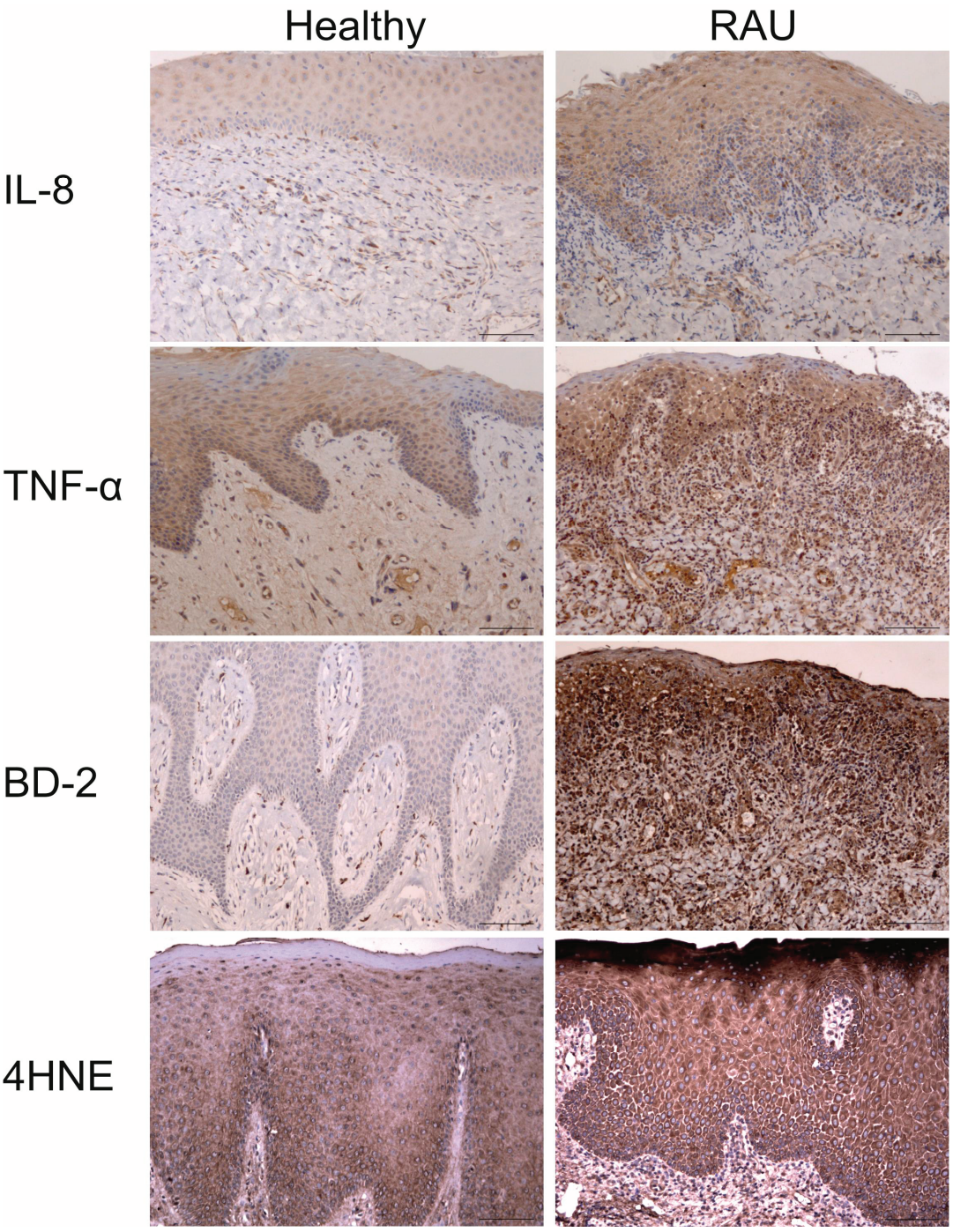

Figure 4. Expression of select chemokine, pro-inflammatory cytokine, antimicrobial peptide, and oxidative stress markers in healthy and RAU oral mucosa. Healthy and RAU oral mucosa were immunostained for IL-8, TNF-α, BD-2, and 4HNE. Scale bar = 100μm.

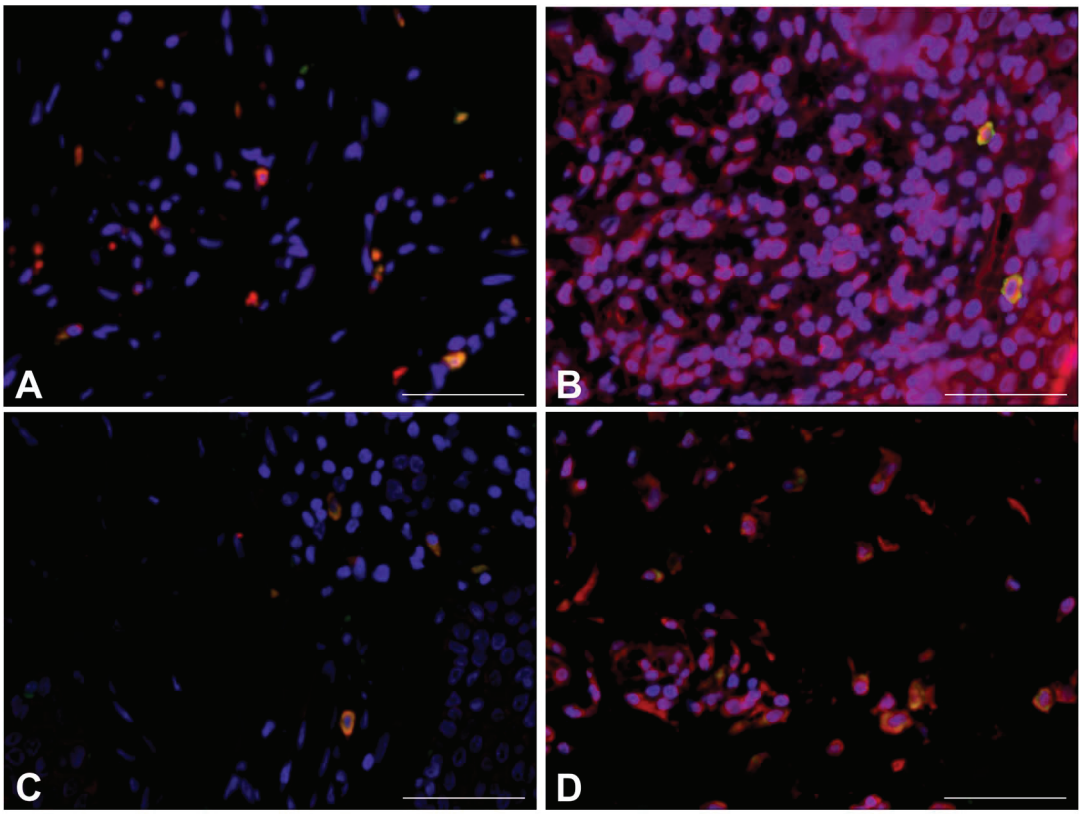

Figure 5. Identification of BD-2-producing cells in healthy and RAU oral mucosa. Healthy and RAU oral mucosa were subjected to immunofluorescence staining for BD-2, MCT, and CD68. Healthy oral mucosa (A & C); RAU mucosa (B & D). BD-2 and MCT (top row); BD-2 and CD68 (bottom row). DAPI (blue), BD-2 (red), MCT (green), CD68 (green), and co-localisation of BD-2 with MCT or CD68 (yellow). Scale bar = 50 μm.

5.2. TLR expression pattern in RAU lesions and healthy control mucosa

TLR expression was studied in the biopsy samples taken from the healthy controls, ulcerated RAUs, and two samples taken from the healthy mucosa of RAU patients. The pattern of TLR distribution is clearly different in RAU lesions compared to that from the control samples (Figure 6).

In the control oral epithelium, most TLRs share the same pattern of expression, being strongly positive at the basal and suprabasal layers of the epithelium. TLR expression decreased towards the superficial layer of the epithelium where it first

disappeared in the middle layer for all TLRs except TLR7, while the superficial layer showed a slight staining for TLRs.

In RAUs, the pattern of expression clearly differed. The basal and suprabasal layers stained most intensively positive for TLRs, but the pattern differed in the other layers (lower, middle, and upper spinal layers) since these layers stained positive for TLRs in RAU.

While most TLRs were quite similar in their pattern of expression in both the control and RAU epithelium, some showed differences since TLR7 expressed in all layers of healthy epithelium, yet remained more strongly positive in RAU. TLR9 was negative in almost all layers for both the control and RAU epithelium.

The two healthy samples taken from RAU patients did not differ from the healthy control mucosa (data not shown).

In the lamina propria, the connective tissue cells (endothelial cells, fibroblasts, and leukocytes) stained positive for all TLRs except for TLR9 (data not shown). Despite the positive stains for most connective tissue cells in both healthy and RAU lesions, the number of cells was higher in RAU compared to that in the healthy controls resulting from leukocyte migration towards the inflamed area.

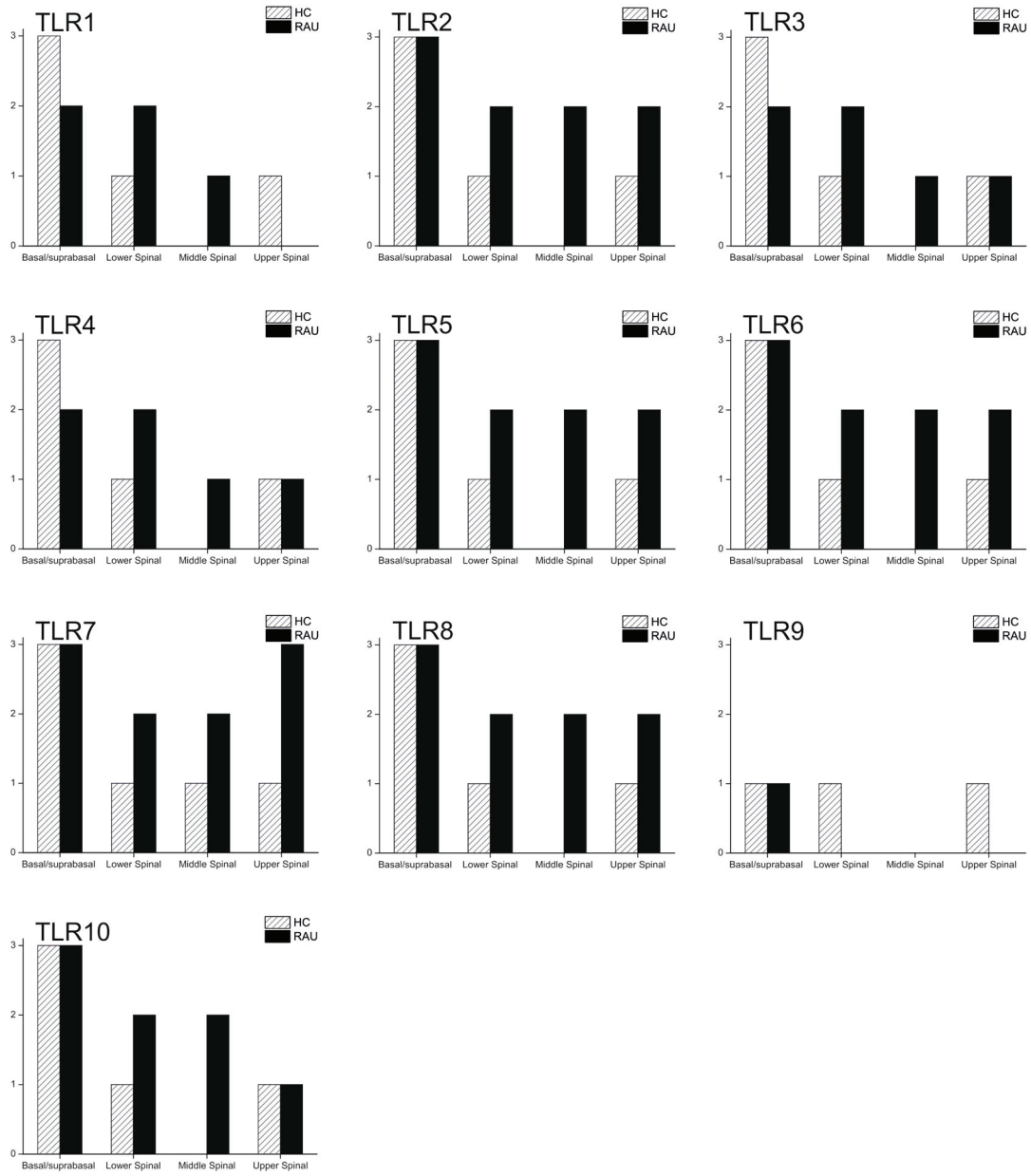

Figure 6. TLR expression in healthy and RAU oral mucosa. Healthy control (HC) and RAU oral mucosa were immunostained for TLR1-10. TLR expression in the epithelium of healthy control and RAU were graded as follows: 0 = negative; 1 = weakly positive; 2 = moderately positive; 3 = strongly positive.

5.3. Functional studies of cultured primary oral keratinocytes, oral keratinocyte SCC-25 cell lines, and primary gingival fibrobasts

Effect of TNF-α and IFN-γ on TLR2 and TLR4 mRNA

We studied the effect of pro-inflammatory cytokines on the expression of TLRs *in vitro*. TNF-α and IFN-γ were selected based on their effect on TLR expression in other cell types. TLR2 and TLR4 were selected as the target receptors since they possess the ability to bind with both PAMPs (as LPS) and DAMPs (as HMGB1).

Neither TNF-α nor IFN-γ alone was able to increase TLR2 mRNA. By contrast, the synergistic effect of TNF-α and IFN-γ significantly up-regulated TLR2 mRNA ($p = 0.01$; Figure 7A). This increase was also shown at the protein level by immunofluorescence staining (Figures 7B–C).

We found a different situation with TLR4. TNF-α did not increase TLR4 mRNA, but IFN-γ alone led to a noticeable increase in the TLR4 mRNA even though it did not reach statistical significance due to a high skewed deviation. Adding TNF-α to IFN-γ did not yield an additional effect on the TLR4 mRNA ($p > 0.05$; Figure 7C). While TNF-α and IFN-γ did not significantly affect mRNA expression, their effect on the protein level was clear (Figure 7E–F).

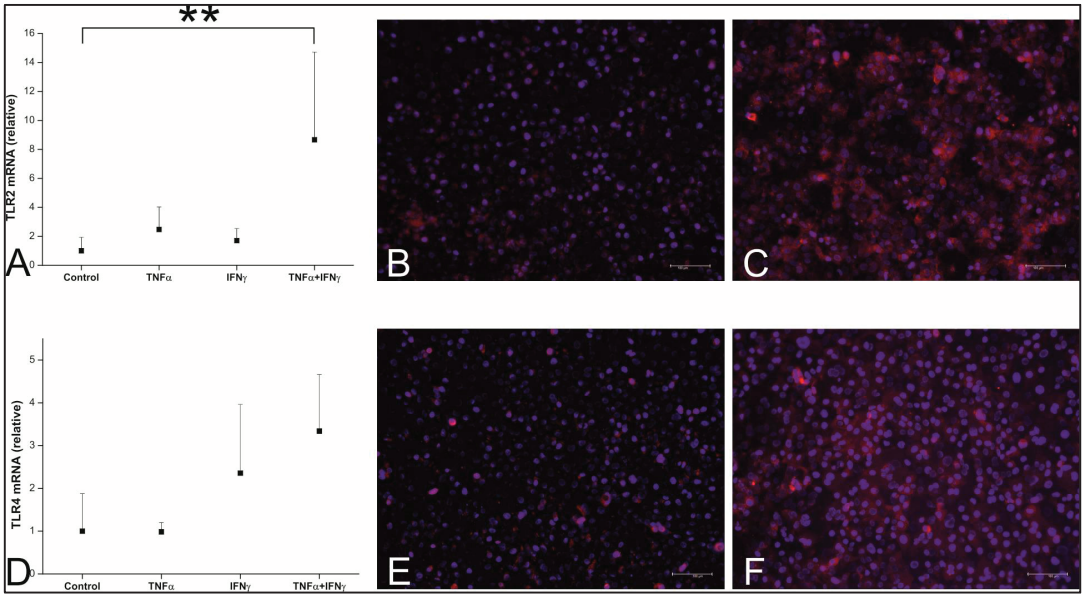

Figure 7. TLR2 and TLR4 expression in controls and TNF-α and IFN-γ stimulated human oral keratinocyte SCC-25 cells. Human oral keratinocyte SCC-25 cell lines were stimulated with 50 ng/ml TNF-α, 50 ng/ml IFN-γ, and both combined for 4 and 24 h, respectively. TLR2 (A) and TLR4 (D) mRNA were measured using qPCR. The TLR2 (B–C) and TLR4 (E–F) protein levels were shown using immunofluorescence staining; control SCC-25 cells (B, E); TNF-α and IFN-γ stimulated SCC-25 cells (C, F). DAPI (blue); TLR2 and TLR4 (red). Scale bar = 50 μm.

Loss of nuclear HMGB1 from SCC-25 after activation with TNF-α

Human oral keratinocytes SCC-25 were cultured on coverslips and stimulated with 50 ng/ml TNF-α for 24 h. In control cells, HMGB1 was found in the nucleus (Figure 8A). Most of the stimulated cells lost their HMGB1 and their nuclei were HMGB1 negative or weakly positive (Figure 8B). The corrected total cell fluorescence for the control SCC-25 cells was significantly higher than that in the stimulated cells ($p \leq 0.0001$, data not given).

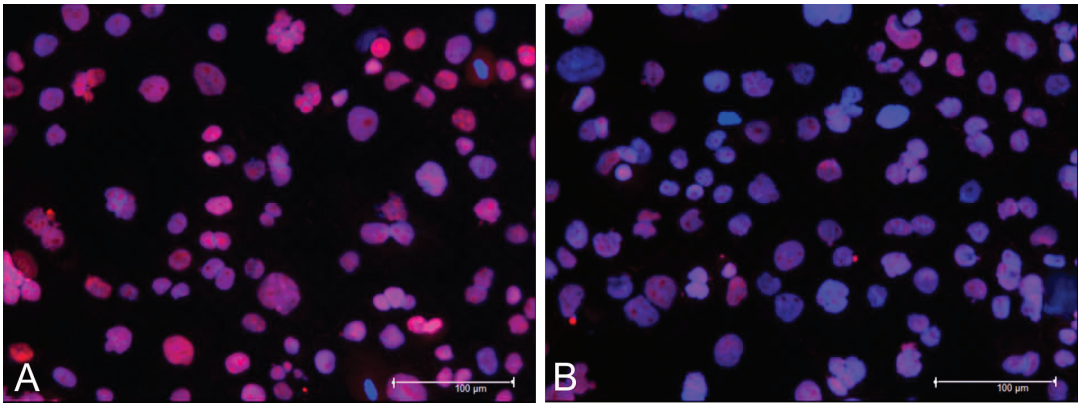

Figure 8. HMGB1 expression in healthy control and TNF-α stimulated human oral keratinocyte SCC-25 cells. Human oral keratinocyte SCC-25 were cultured on coverslips and stimulated with 50 ng/ml TNF-α for 24 h. Cells were subjected to immunofluorescence staining with HMGB1. Non-stimulated cells (A); stimulated cells (B). DAPI (blue); HMGB1 (red). Scale bar = 100 µm.

Stimulation of SCC-25 cells and fibroblasts with different forms of HMGB1

Here, 1000 ng/ml all-thiol HMGB1, 1000 ng/ml disulfide-HMGB1, 50 ng/ml IFN-γ, or a combination of disulfide-HMGB1 and IFN-γ were used to stimulate SCC-25 cells for 8 and 24 h. None of the stimulants, neither alone nor in combination with each other, up-regulated IL-8, IL-6, or TNF-α mRNA (data not given).

Gingival fibroblasts, another oral mucosal cell type, were tested in the HMGB1 functional assay. Similar to the results from SCC-25 cells, none of the HMGB1 forms up-regulated IL-8, IL-6, or TNF-α mRNA (data not given).

In order to check the functionality of TLR4, a low dose of LPS (10 ng/ml) was used to stimulate gingival fibroblasts alone or in combination with disulfide-HMGB1. Even with such a low concentration, LPS was able to up-regulate IL-8 ($p \leq 0.0001$) and IL-6 ($p \leq 0.001$), but not TNF-α. The combination of disulfide-HMGB1 with LPS did not yield an additional up-regulation compared to LPS alone (Figure 9).

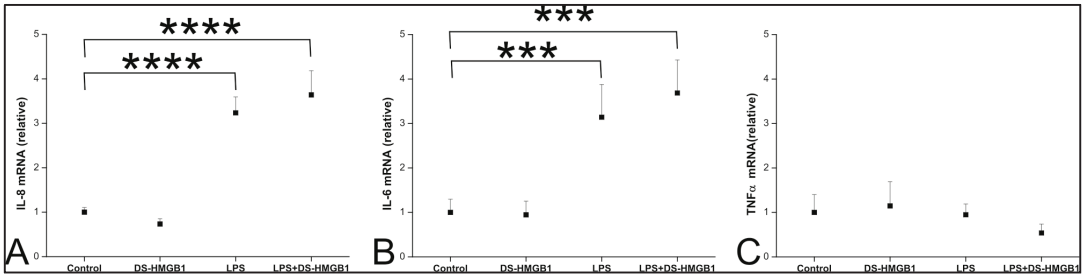

Figure 9. Effect of LPS and disulphide-HMGB1 (DS-HMGB1) on the expression of IL-8, IL-6, and TNF-α in human gingival fibroblasts. Gingival fibroblasts were stimulated with 1000 ng/ml DS-HMGB1, 10 ng/ml LPS, and both for 24 h. IL-8 (A), IL-6 (B), and TNF-α (C) mRNA were measured using qPCR.

Stimulation of SCC-25 cells with self-DNA

SCC-25 cells were stimulated using 50 ng/ml self-DNA extracted from SCC-25 cells for 8 and 24 h. While IL-8 mRNA was not affected by self-DNA (Figure 10A), TNF-α mRNA was significantly up-regulated after 24 h of stimulation ($p = 0.02$; Figure 10B).

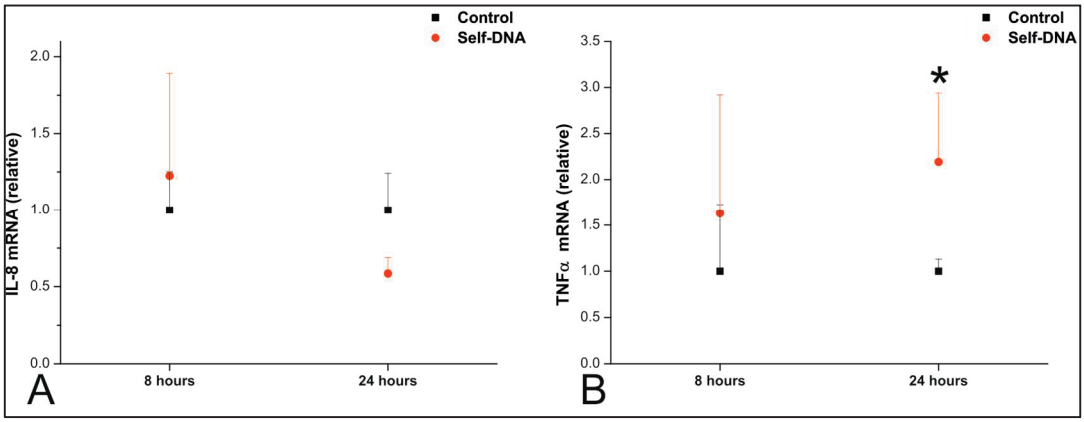

Figure 10. Effect of self-DNA on the expression of IL-8 and TNF-α in human oral keratinocyte SCC-25 cells. Human oral keratinocyte SCC-25 cells were stimulated with 50 ng/ml self-DNA for 8 and 24 h. IL-8 (A) and TNF-α (B) mRNA were measured using qPCR.

Effects of IL-17C on human primary oral keratinocytes

Human oral keratinocytes were stimulated using 100 ng/ml IL-17C for 4 and 12 h. After the first 4 h, we found a slight increase in the IL-8 and TNF-α mRNA from the stimulated cells compared with the control cells, but the difference was not statistically significant. After 12 h, IL-17C significantly up-regulated TNF-α mRNA ($p = 0.03$). We found a similar increase in the IL-8 mRNA, but the difference was not statistically significant ($p > 0.05$; Figure 11).

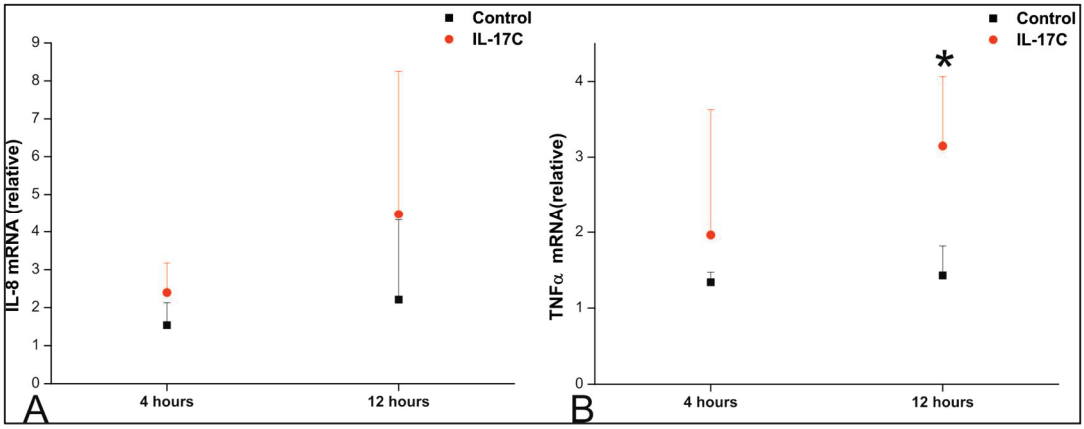

Figure 11. Effect of IL-17C on the expression of IL-18 and TNF-α in primary human oral keratinocytes. Human primary oral keratinocytes were stimulated with 100 ng/ml IL-17C for 4 and 12 h. IL-8 (A) and TNF-α (B) mRNA were measured using qPCR.

Synergistic effect of TNF-α and IL-17C in the presence or absence of oxidative stress on the production of BD-2 from SCC-25 cells

SCC-25 cells were stimulated using 100 ng/ml IL-17C, 10 ng/ml TNF-α, or both together. While IL-17C did not affect BD-2 mRNA, TNF-α significantly up-regulated BD-2 mRNA ($p < 0.0001$). This up-regulation increased further when IL-17C was added together with TNF-α ($p < 0.0001$). The BD-2 mRNA level was significantly higher in TNF-α and IL-17C stimulated cells than when using TNF-α alone ($p = 0.02$; Figure 12A).

Oxidative stress was induced in the cultured cells by adding vitamin K3. Vitamin K3 did not affect the BD-2 mRNA neither in the 'unstimulated' control cells nor in the 'TNF-α and IL-17C stimulated' activated cells (Figure 12B).

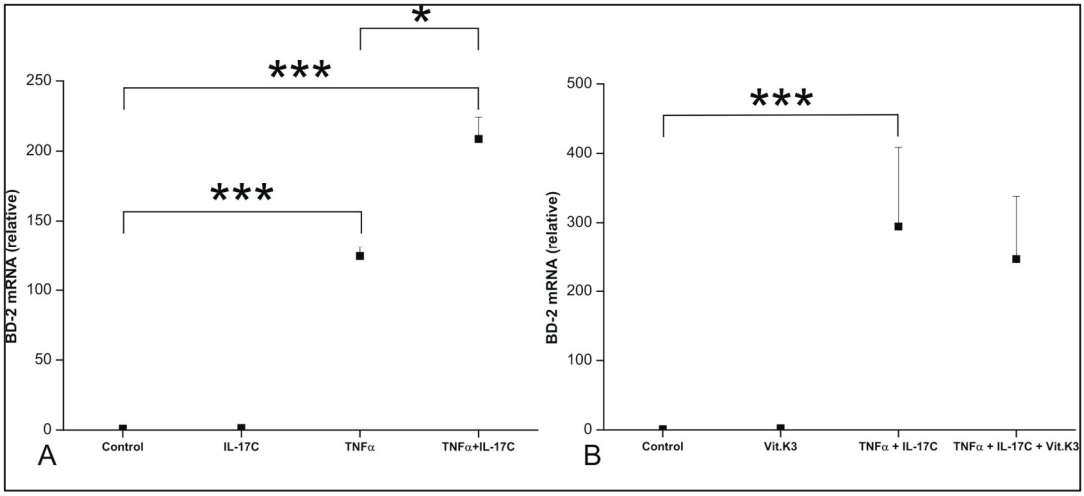

Figure 12. Effect of TNF-α and IL-17C on the expression of BD-2 in healthy control and stressed human oral keratinocyte SCC-25 cells. Human oral keratinocyte SCC-25 cells were stimulated with 100 ng/ml IL-17C, 10 ng/ml TNF-α, and both in the absence (A) and presence of vitamin (vit) K3 for 24 h. BD-2 mRNA was measured using qPCR.

Table 11. Summary of mRNA and protein expression of the molecules studied in the oral epithelium of control and RAU samples and cultured primary oral keratinocytes, squamous cell carcinoma SCC-25, and primary gingival fibroblasts.

Molecule	Tissue sample		Cultured cells						Study
	Control	RAU	HOK		SCC-25		Gingival fibroblast		
	Protein	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein	
4HNE	+	+							IV
BD-2	-/+	+			+				IV
Caspase-3	-	+							I
HMGB1	+	+				+			I
IL-6					+		+		I
IL-8	+	+	+		+		+		I, III
IL-17A	-	-	-						III
IL-17C	+	+	+						III
IL-17RA			+						III
IL-17RC			+						III
TLR1	+	+							II
TLR2	+	+			+	+			I, II
TLR3	+	+							II
TLR4	+	+			+	+			I, II
TLR5	+	+							II
TLR6	+	+							II
TLR7	+	+							II
TLR8	+	+							II
TLR9	+	+							II
TLR10	+	+							II
TNF-α	+	+	+		+		+		I, III

6. Discussion

6.1. Epithelial cell death in RAU: apoptosis or necrosis?

Ulceration may be defined as the full thickness loss of epithelium (Sonis, 2007). This loss can be caused either by the sloughing of the epithelium resulting from trauma such as a traumatic ulcer or by epithelial cell death caused by necrosis or apoptosis. In earlier studies of RAU, the mechanism for oral epithelial cell death received relatively little attention. In our research on the role of apoptosis in RAU, we only found one study claiming that apoptosis plays a role in RAU. In that study, an electron microscope was used to identify the features of apoptosis in epithelial cells including nuclear and cytoplasmic shrinkage (Honma et al., 1985).

In our study, we used cleaved caspase-3 and TUNEL staining to show apoptotic cells in RAU. Our results agreed with previous studies on apoptosis. The oral epithelial cells stained strongly positively in RAU lesions and completely negatively in healthy controls. The pattern of caspase-3 expression was not the same through all layers of the RAU epithelium since it was weak in the basal layer, very strong in the middle layer, and strong in the superficial layer. Such an expression may indicate that the epithelial cell death begins from the superficial layer resulting from a strong and sudden triggering factor, decreasing down to the basal layer.

Caspase-3 was not expressed in the control epithelium, agreeing with proven phenomena. This suggests that in healthy oral mucosa oral epithelial cells flow from the deep layer to the superfacial layer where they detach from the undelying layer of the oral epithelium. They then undergo apoptosis as a result of the loss of cell–cell contact (Loro et al., 2005).

Our results were unexpected, since apoptosis is not normally accompanied by inflammation given that all cellular contents and DAMPs are preserved inside apoptotic bodies, and then cleared by macrophages, contrary to what we find in RAU where the ulcer is surrounded by an inflamed halo area (Natah et al., 2004).

This discrepancy may be explained by one of two theories: secondary necrosis and necroptosis. The first theory, secondary necrosis, depends on whether apoptotic bodies remain in the tissue for a long time, when they enter secondary necrosis with features similar to necrosis. While macrophages are quite efficient in engulfing cellular debris, this efficiency may not be suffiicent to handle the huge number of apoptotic keratinocytes which result from massive transepithelial apoptosis. As a result of this delay in the elimination of apoptotic bodies, they may result in secondary necrosis and the release of DAMPs, such as self-DNA and HMGB1 (Abdulahad et al., 2010). The role of secondary necrosis and the release of alarmins were found in other epidermal diseases, such as lupus erythematosus. Here, ultraviolet radiation was found to trigger lupus, activating apoptosis cascades in keratinocytes, potentially leading to huge numbers of apoptotic keratinocytes. Macrophages failed to deal with all apoptotic bodies, potentially leading to secondary necrosis and the release of HMGB1 (Abdulahad et al., 2010; Baumann et al., 2002).

The second theory suggests a new mechanism of cell death which includes necroptosis or programmed necrosis and is also associated with inflammation. On the one hand, the necroptosis theory may be applied to RAU since necroptosis may be initiated after the activation of TLRs as TLR3 and TLR4 or by TNF-α, where both TLRs and TNF-α are overexpressed in RAU (Shalini et al., 2015). On the other hand, the biggest drawback of this hypothesis in this context is that it is associated with the inactivation of caspase-3, which is activated in RAU epithelium (Gunther et al., 2011; Han et al., 2015; Li et al., 2011). Further studies on other markers of necroptosis in RAU are needed.

6.2. HMGB1: an increase in the expression in RAU but has no clear function

HMGB1 is regarded as one of the most important DAMPs (Wang et al., 1999). In healthy oral epithelium, HMGB1 is found in the perinuclear area of the oral

epithelial cells in the basal layer, which is the only active layer and the only layer which allows for mitosis. The expression of HMGB1 in this layer is understandable since HMGB1 is known to play an important role during cell mitosis through transcription, replication, and cellular differentiation (Stros, 2010). The other layers of oral epithelium were HMGB1 negative. Since all cells have a specific amount of HMGB1, then the possible explanation for this negative staining of HMGB1 relies on it being tightly bound inside the nucleus. This results from its lack of function in these cells due to the loss of its ability in cell division, rendering it unrecognisable to the antibody (Heinola et al., 2010).

In RAU, HMGB1 was found in all layers of the epithelium. It was expressed more in the cytoplasm and extracellularly. This dramatic change in the expression of HMGB1 in the oral epithelium may be explained either by its undergoing apoptosis since HMGB1 is known to first translocate to cytoplasm and then to extracellular tissue during apoptosis (Abdulahad et al., 2010; McNamara et al., 2010). This may also result from the activation of oral epithelial cells stimulating SCC-25 using TNF-α leading to a loss of HMGB1 from the nuclei. A similar expression of HMGB1 was found in murine-injured skin compared with a healthy control (Straino et al., 2008).

Our results showing the presence of HMGB1 expression in all epithelial layers of RAU lesion is of importance as TLR4, the receptor for HMGB1 (Venereau et al., 2012), has the same pattern of expression in RAU. This indicates that these cells have the ability both to release HMGB1 and also to respond to it.

HMGB1 plays an important role in inflammation through stimulating macrophages to produce chemokines and pro-inflammatory cytokines (Venereau et al., 2012). However, very little is known about the effects of HMGB1 on oral epithelial cells, especially in terms of the production of chemokines and pro-inflammatory cytokines. Therefore, we studied the effect of different forms of HMGB1 on oral epithelial cells. Contrary to our expectations, none of these forms increased the

mRNA of IL-8, IL-6, and TNF-α. Next, we added IFN-γ to the medium with disulfide-HMGB1, since previous studies showed that LPS shares the same receptors, TLR2 and TLR4, and requires IFN-γ to stimulate oral epithelial cells (Uehara et al., 2002). Even with IFN- γ, HMGB1 did not affect the oral epithelial cells.

In the next step, we chose gingival fibroblast, another type of cell present in oral mucosa, which may also play a role in aggravating inflammations in RAU lesions. Similar results were obtained forfibroblasts and oral epithelial cells. Then, we checked the functionality of TLR4 by stimulating fibroblasts using a low concentration of LPS. Using a concentration of 10 ng/ml, which is 100 times less than the concentration used for HMGB1, LPS increased IL-8 and IL-6 mRNA, suggesting that the problem of HMGB1 stimulation was unrelated to its receptor, but attributed to the protein itself.

Since this is the first report regarding the inability of HMGB1 to affect oral keratinocytes in terms of the production of chemokines and pro-inflammatory cytokines, we recommend carrying out further experiments in future using another cell line and attempting to find a synergistic effect between HMGB1 and other proteins. If further experiments yield negative results that support ours, then it may be that HMGB1 stimulates inflammatory cells such as macrophages, but not other non-professional immune cells such as keratinocytes.

6.3. Self-DNA: another alarmin in RAU

During secondary necrosis, several molecules are passively released from secondary necrotic cells to the extracellular space (Abdulahad et al., 2013). First, we discussed HMGB1 as an example of proteins which work as an alarmin. Here, we took self-DNA as another example for DAMPs. Self-DNA was extracted from oral keratinocytes and used to stimulate the same cells in order to simulate necrosis *in vivo*. Oral keratinocytes were stimulated with self-DNA without needing a

transfecting agent since keratinocytes carry the ability to take up DNA using macropinocytosis (Basner-Tschakarjan et al., 2004). While we are unsure which receptor is responsible for binding to self-DNA, the effect of this binding was quite clear through the up-regulation of TNF-α mRNA. IL-8 mRNA was not affected by self-DNA, demonstrating the specificity of this stimulation.

The ability of oral keratinocytes to respond to their own DNA stimulation may partially support our hypothesis about identifying oral epithelial cells as inflammatory cells in RAU. These cells may play a significant role in inducing inflammation in the inflammatory halo area around the ulcer edge by secreting intracellularly confined danger signals which work as as alarmins in pathological conditions such as RAU. These alarmins may stimulate adjacent epithelial cells at the ulcer margins to produce pro-inflammatory cytokines and exacerbate the inflammation.

6.4. TLR up-regulation in RAU

Oral epithelium is the first line of defence in the oral mucosa. Due to its physical characteristics, it provides physical protection against microbial invasion, irritation, and trauma (Beklen et al., 2008). In addition, oral epithelial cells are equipped with 10 TLRs, which have the ability to recognise microbes through their PAMPs (Ali et al., 2008). Such recognition leads to the immediate production of chemokines, pro-inflammatory cytokines, and antimicrobial peptides. Rapid reaction provides immediate defence against the microbes before the arrival of inflammatory cells. In healthy oral epithelium, TLR receptors were found only in the basal and suprabasal layers. This distribution provides protection against the continuous activation of TLRs, since the superficial layer of oral epithelium is in direct contact with commensal oral microbes, which share some PAMPs with pathogenic microbes. As a result of this distribution, only microbes with the ability to invade into the suprabasal and basal layers of epithelium can activate TLRs.

TLRs are distributed through all layers of oral epithelium in RAU (including the superficial layer) in contrast to healthy epithelium. As a result of this distribution, TLRs are activated by penetrating microbes throughout the ulcer area. The ulcer area provides easy access to microbes, as well as to commensal and pathogenic microbes in the oral cavity which have direct contact with the superficial layer of the oral epithelium. The chemokines produced from activated epithelial cells lead to the recruitment of inflammatory cells, which also have TLRs. Such cells form a third line of defence (Uehara et al., 2002).

The distribution of TLRs in non-ulcerated RAU epithelium mimicked healthy epithelium, indicating that this up-regulation of TLRs is not a constant feature, but a part of the inflammatory process associated with RAUs. Differences in the TLR distribution between a RAU lesion, periodontitis, and candidiasis show that this distribution is affected by the type of inflammation (chronic or acute), the type of cytokines produced in the area, and the type of PAMPs and DAMPs associated with the lesion (Ali et al., 2008; Beklen et al., 2008).

Several cytokines, such as transforming growth factor (TGF)-α, TNF-α, IFN-γ, IL-1β, and G-CSF, and PAMPs such as poly (I:C), LPS, LTA, and soybean lipoxygenase (SLO), may up-regulate TLRs as TLR1, TLR2, TLR3, TLR5, and TLR9 in different types of cells, including endothelial cells, small airway epithelial cells, and skin keratinocytes (Joo et al., 2011; Lew et al., 2009; Miller et al., 2005; Ritter et al., 2005; Satta et al., 2008).

In this project, we selected two cytokines expressed in RAU mucosa and tested their effect on TLR2 and TLR4 (Al-Samadi et al., 2014; Buno et al., 1998). TLR2 and TLR4 carry the ability to bind with both PAMPs (e.g., LPS) and DAMPs (e.g., HMGB1). Our results clearly showed that neither TNF-α nor IFN-γ alone had an effect on TLR2 expression. However, when SCC-25 cells were exposed subjected to both simultaneously, the TLR2 expression was significantly up-regulated, pointing to the importance of cell priming with IFN-γ in oral keratinocyte SCC-25

cells. TLR4 mRNA behaves differently since IFN-γ alone was sufficient to achieve a noticeable increase in TLR4 mRNA although not statistically significant. We also found no synergistic effect between TNF-α and IFN-γ. Our results partially agreed with Uehara et al., who claimed that IFN-γ alone up-regulates TLR4 (Uehara et al., 2002). At the same time, our results contradict those of Uehara et al. who showed that IFN-γ alone up-regulates TLR2 (Uehara et al., 2002). Such a discrepancy may stem from the fact that they stimulated the oral epithelial cells for three days in their experiment, which is a relatively long time, during which oral keratinocytes may produce TNF-α. Previous studies found that these cells include the ability to continuously produce TNF-α in a cell culture media (Formanek et al., 1999). The accumulation of TNF-α in the medium may act in a synergistic manner with IFN-γ. To overcome this problem, we stimulated cells for only 4 h. Even with such a short-duration stimulation, TLR2 was significantly up-regulated and TLR4 was noticeably up-regulated. Such a quick up-regulation may provide an indication of how fast the inflammatory process may be initiated in RAU. These results were also supported by the increase in the TLR2 and TLR4 protein levels in TNF-α and IFN-γ stimulated SCC-25 cells as detected by immunofluorescence staining.

6.5. IL-17C: an active player in RAU

IL-17C is a relatively new member of the IL-17 family. It shares some characteristics with other IL-17 family members even though it is not produced by Th17 cells (Iwakura et al., 2011). Since we concentrated here on the role of oral epithelial cells in RAU, we took IL-17C as a suitable example of pro-inflammatory cytokines. Our selection was dependent on the fact that epithelial cells are the primary producers of IL-17C. Previous findings showed that epithelial cells may be activated by TLR ligands to produce IL-17C (Ramirez-Carrozzi et al., 2011). The regulation of IL-17C by TLRs is important in RAU lesions, as we demonstated with TLRs up-regulation and exposure to a variety of PAMPs and DAMPs.

Our qPCR results proved that oral epithelial cells carry the ability to produce IL-17C, but not IL-17A. These cells were also IL-17RA and IL-17RE mRNA positive, meaning that they are not just major producers of IL-17C, but that they can respond to it.

Immunostaining of oral mucosa from healthy controls and RAU lesions confirmed our results of qPCR since IL-17C was weakly positive in healthy epithelium and strongly positive in RAU. Our first explanation regarding the presence of IL-17C in healthy epithelium stems from the tissue being taken from oral mucosa during wisdom tooth extraction. Furthermore, this mucosa may have subclinical inflammation even though we selected these samples to be clinically and histologically healthy. Our conclusions have shifted, however, after Johnston et al. reported the positive staining of IL-17C in healthy skin keratinocytes (Johnston et al., 2013). The presence of IL-17C in healthy oral epithelium may provide baseline protection against harmful conditions. Given these results, we argue that studies on the role of IL-17C in keratinocyte proliferation and specifically the balance between epithelial cell apoptosis and proliferation are required.

In RAU, the expression of IL-17C in oral epithelial cells increased significantly. Such an increase is not surprising given that TLRs are up-regulated and expressed in all layers of the epithelium, making the cells more responsive to stimulants. Additionally, the oral cavity is not a sterile field and is full of several PAMPs.

The overexpression of IL-17C may induce a severe and self-amplifying inflammatory cycle as we demonstrated here. IL-17C increased the production of TNF-α significantly and TNF-α is known as an inducible agent for IL-17C (Johansen et al., 2011). Our cell culture results were supported by the results from clinical lesions of RAU patients given that the immunostaining of healthy and RAU oral mucosa revealed a significant increase in TNF-α in the oral epithelium of RAU when compared with healthy controls. In addition to oral epithelial cells,

neutrophils highly infiltrated in both epithelium and lamina proparia to provide a second source of IL-17C.

We studied IL-17A here as a negative control. Oral epithelial cells were IL-17A negative in both the control and RAU lesions. Some cells in the lamina proparia stained positive, forming an internal positive control, in both the control and RAU lesions. Based on the morphology of the cells, we hypothesised that these cells are mast cells. Double immunofluorescence staining of MCT and IL-17A confirmed our hypothesis. This also supports our assumption that mast cells represent the primary producers of IL-17A in oral mucosa. Similar results have also been reported for psoriasis, rheumatoid arthritis, and esophageal squamous cell carcinoma (Kouri et al., 2014; Lin et al., 2011; Wang et al., 2013).

6.6. Oxidative stress in RAU

Oxidative stress has been studied intensively as a possible cause for RAU. All of these studies investigated the total oxidant/antioxidant status in the plasma and saliva of RAU patients comparing them with healthy controls. Here, we studied the oxidative stress in oral epithelium for the first time. Indeed, 4HNE, an endproduct of lipid peroxidation, was expressed in both the control and RAU samples, but with no clear difference. Our results prove interesting given that the superficial layer of the oral epithelium was strongly positive for 4HNE compared with the control which was negative. Such an expression may suggest that oxidative stress begins in the superficial layer as a result of a strong initiating stimulus moving into the deeper layers. This suggestion is also supported by the expression of caspase-3, which expresses strongly at the superficial and middle layer and quite weakly at the basal layer.

To test if the oxidative stress affects the mucosal immune defence, we induced oxidative stress in a cell culture by adding vitamin K3 to the SCC-25 cell culture medium. Stressed and non-stressed SCC-25 cells behaved similarly in term of BD-

2 expression. These results indicate that oxidative stress has no significant effect on the defence mechanism of oral epithelial cells, which may also explain the strong expression of BD-2 in RAU.

6.7. Protection of the ulcer area by BD-2

As mentioned before, the oral mucosa of RAU lesions subjected to several types of microbes have direct access to the area through the ulcer. The PAMP–TLR interaction may initiate a self-amplificatory cycle in RAUs. Since RAU is a self-healing ulcer taking up to 14 days to heal spontaneously, this suggests that the cycle may be interrupted somewhere (Belenguer-Guallar et al., 2014), which may serve as the first step in the healing process.

The rapid healing of an acute inflammation such as RAU may indicate that oral mucosa carries a strong defence mechanism against microbial invasion. Antimicrobial peptides are known to be very efficient microbicidal agents which work similarly to some synthetic antibiotics. Between these peptides, beta defensins represent the most important peptides in the oral epithelium. Beta defensins are divided into two types: non-inducible (continuous release) beta defensins such as BD-1 which express even in healthy epithelium and provide continuous protection to oral mucosa, and inducible beta defensin such as BD-2 and BD-3 (Gursoy and Kononen, 2012).

As a result of an acute inflammation in and around the ulcer area, finding a high expression of both the oral epithelium and inflammatory cells infiltrating the lamina proparia of a RAU lesion is unsurprising.

BD-2 may be induced by several cytokines and PAMPs (Kanda et al., 2011). In RAU, the reason for the increase in BD-2 production remains poorly understood. In our study, we selected two cytokines, TNF-α and IL-17C, which we already found up-regulated in RAU epithelium. In contrast to epidermal keratinocytes in which IL-17C stimulates keratinocytes to produce BD-2 (Ramirez-Carrozzi et al., 2011),

BD-2 mRNA expression did not increase after exposing the oral keratinocyte cell line to IL-17C. Our experiments demonstrated the important role of TNF-α in the immune defence of the epithelium given that TNF-α increased the BD-2 mRNA more than 100-fold. Since it is already known that IL-17A can work with TNF-α in a synergistic manner (Moniaga et al., 2011; Simanski et al., 2013), we tested this for IL-17C. BD-2 mRNA expression increased significantly after simultaneous stimulation with TNF-α and IL-17C as compared with TNF-α only. The similar synergistic behavior of IL-17A and IL-17C with TNF-α may be explained by the signaling pathways of these two cytokines.

BD-2 positive cells in the lamina proparia of RAU lesions were significantly higher than in healthy control cells. This increase resulted from the migration of inflammatory cells to the inflamed area. In healthy lamina proparia, macrophages and mast cells, which are always present in oral mucosa, were identified as the primary source of BD-2. In RAU, the situation differs due to the arrival of inflammatory cells which are BD-2-positive cells.

7. Conclusions

This study focused on the behavior of oral epithelial cells in RAU lesions, especially in terms of oral epithelial cell death, TLR expression, the response of oral epithelial cells to pro-inflammatory cytokines and DAMPs, and the production of chemokines, pro-inflammatory cytokines, and antimicrobial peptides. Our main findings are as follows:

A. Oral epithelial cells in RAU stained positive for apoptosis markers caspase-3 and TUNEL. These results indicate that the death of oral epithelial cell begins with a strong triggering factor inducing apoptosis. In turn, transepithelial oral epithelial cell apoptosis leads to the sloughing of the oral epithelium and ulcer formation. The association of inflammation with the apoptotic death of the oral epithelial cells in RAU may be returned to secondary necrosis or necroptosis.

B. TLRs are up-regulated in RAU lesions and expressed in all layers of the epithelium, contrary to healthy controls where they are expressed only in the basal and supra basal layers of the epithelium. This up-regulation may increase the response of oral epithelial cells to PAMPs and DAMPs. TLR up-regulation may be a result of the underlying inflammation since inflammatory cytokines (TNF-α and IFNγ) were able to increase the expression of TLR2 in SCC-25 cells.

C. DAMPs (such as self-DNA) and pro-inflammatory cytokines (such as IL-17C) were able to stimulate oral epithelial cells to produce TNF-α. IL-8, TNF-α, and IL-17C were strongly expressed in RAU oral epithelium and were significantly higher in RAU epithelium compared with the control epithelium. The activation of oral epithelial cells and the secretion of TNF-α point to the ability of these cells to behave like pro-inflammatory cells in RAU.

D. The antimicrobial peptide BD-2 is strongly expressed in RAU epithelium and its expression is significantly higher in RAU epithelium compared with healthy controls. Such an increase in this expression level may result from the synergistic effect between TNF-α and IL-17C.

As a final conclusion based on our results, we argue that the onset of RAU may be precipitated by a strong triggering factor inducing apoptosis and ulcer formation in the oral epithelium. DAMPs are released from dying cells and up-regulate inflammation through stimulation of the oral epithelial cells to produce chemokines and pro-inflammatory cytokines. Pro-inflammatory cytokines first up-regulate TLRs in the oral epithelium making cells more responsive to PAMPs and DAMPs. Second, they stimulate oral epithelial cells to produce antimicrobial peptides to kill microbes and initate the healing process.

Due to the lack of memory, a new trigger leads to recurrence and, under each cycle, the same sequence of events occurs. This RAU cycle is illustrated in Figure 13.

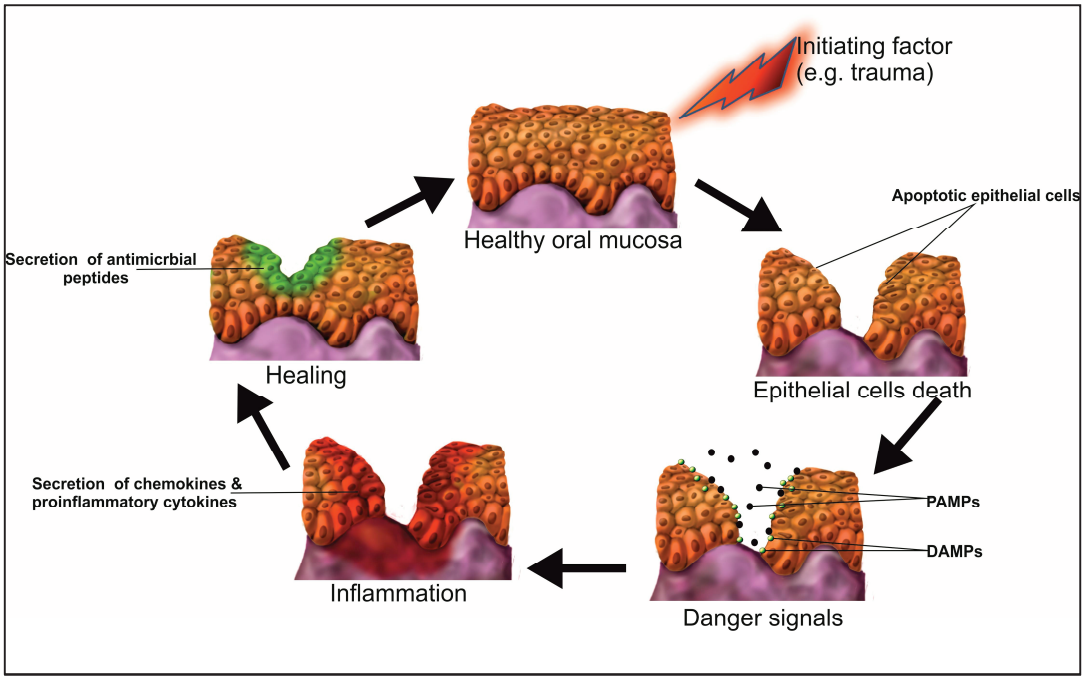

Figure 13. Illustration of the RAU cycle.

8. **Acknowledgments**

The studies forming this PhD thesis were carried out at the Institute of Clinical Medicine, Department of Medicine and Institute of Biomedicine, Department of Anatomy at the University of Helsinki between 2011 and 2015.

I would like to first thank God for everything I have in this life.

My sincerest gratitude goes to a person who unfortunately is no longer with us, but whose words will live on forever: my supervisor Professor Yrjö Konttinen. Professor Konttinen was not just a supervisor, but a surrogate father and friend to all group members. His love and commitment to his work will persist in my memory and will always push me onward and further.

I would also like to thank Dr. Ritva Häyrinen-Immonen for serving as my second supervisor, and helping me throughout my PhD programme.

I am grateful to Professors Jukka Meurman and Stina Syrjänen for agreeing to be official pre-examiners. Their constructive comments proved quite valuable and markedly improved this thesis. In addition, I am thankful for Professor Ilmo Leivo for kindly accepting my invitation to serve as my dissertation opponent and for Professor Timo Sorsa for kindly accepting my invitation to serve as my dissertation custos.

I wish to thank Professor Kari Eklund and Docent Professor Dan Nordström for their assistance at a very critical time during my PhD research.

All of this work would not have happened without the assistance of my fantastic co-workers from the TULES group, who spent their precious time helping me at various stages. Without a doubt, the biggest thanks goes to my closest friend, Abdulhakim Salem, for the amazing time we spent together and for the many discussions we had on various topics, as well as for our friendship, which will never fade. I am thankful to my friend and co-worker Goncalo Barreto for the lovely lunches and for his company following football matches. I also extend my thanks to Agata Drozd for her exceptional help and hard work on the first article of

my PhD thesis. I am also delighted to work with an expert researcher, Mari Ainola; her insight proved invaluable both on this thesis as well as in other projects. I would like to extend my thanks to my co-worker and the western blotting expert in our group, Vasili Stegajev, for all of his kind assistance. And, I am thankful to Vesa-Petteri Kouri for his help and limitless ideas that helped me on many occasions throughout my doctoral research. I am also thankful to Nina Trokovic and Liisa Virkki for their help and advice in organizing the thesis manuscript and various logistics. My gratitude also extends to all recent and former members of the TULES group including Antti Soininen, Dyah Listyarifah, Eemeli Jämsen, Eija Kaila, Emilia Kaivosoja Hanna Mari, Heidi Poon, Jaakko Levón, Jukka Pajarinen, Juri Olkkonen, Maaria Kärsin, Paulina Porola, Tarvo Sillat, Xia Han , Yan Chen, and Yuya Takakubo.

I am also grateful to Professor Jarkko Hietanen for his help and advice on these studies.

And, I would also like to thank my friend Mohamed El Missiry for his drawings, which I have used in my PhD thesis.

Many thanks as well to Vanessa Fuller for the excellent language editing of my PhD thesis.

My friends, who are too numerous to mention by name, added much to my life during this period. Thanks to all of my friends with whom I spent much-needed time outside the lab; I am honoured to have you in my life.

This work would not have been possible without the financial support received from Finska Läkaresällskapet, the Finnish Dental Society Apollonia, the Centre for International Mobility (CIMO), Sigrid Juselius Stiftelse, the ORTON Invalid Foundation, Orion Farmos, the Academy of Finland, and HUS erva grants.

I would like to thank all those involved in the Doctoral Programme in Oral Sciences (FINDOS) for their help and for allowing me (and my fellow graduate

students) to expand my knowledge through the organisation of the FINDOS video seminars.

Whatever I write here will be insufficient to thank the family who helped me immeasurably to settle down in Finland when I first arrived and continue to help me today. I am profoundly grateful to the family of Professor Zuhair Al-Obaidi, my aunt Salsal Al-Obaidi, and my dearest cousin Marwan. I am proud to have a second family in my life who treats me as if I was their son.

Undoubtedly, the deepest gratitude is reserved for my family, particularly my parents who sacrificed much from the day I was born until now simply to witness me succeed in life. A very special thanks goes to my dearest sisters, Sarah and Nourah, for their kind support not only during my PhD, but throughout life. I also would like my nieces (Dema, Aaya, Mariam, and Rashad) to know how much I miss them.

9. **References**

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